United States Patent [19]

Markussen et al.

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[54] **DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN**

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[73] Assignee: **Novo Industri A/S, Bagsvaerd, Denmark**

[21] Appl. No.: **739,123**

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Feb. 8, 1985 [DK] Denmark 582/85

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[52] U.S. Cl. 530/303; 435/172.3; 435/69.4; 435/320; 435/252.33; 435/942; 435/256; 536/27; 935/13; 935/69

[58] Field of Search 435/253, 172 3, 681, 435/70, 320, 940, 252.33, 256, 942; 536/28, 27; 530/303, 808; 935/2, 17, 18, 13, 29, 40, 69

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,343,898	8/1982	Markussen	435/70
4,431,740	2/1984	Bell	435/252.33
4,440,859	4/1984	Rutter et al.	435/172.3
4,615,974	10/1986	Kingsman et al.	435/172.3

FOREIGN PATENT DOCUMENTS

146482B	10/1980	Denmark .
0006694	1/1980	European Pat. Off. .
0037255	10/1980	European Pat. Off. .
0037723	10/1981	European Pat. Off. .
0040466	11/1981	European Pat. Off. .
0055945	7/1982	European Pat. Off. .
0060057	9/1982	European Pat. Off. .
0068701	1/1983	European Pat. Off. .
0070632	1/1983	European Pat. Off. .
0090433	10/1983	European Pat. Off. .
0121884	10/1984	European Pat. Off. .

OTHER PUBLICATIONS

Greene et al, Methods in Enzymology, vol. XLVII, 1977, pp. 170–172.

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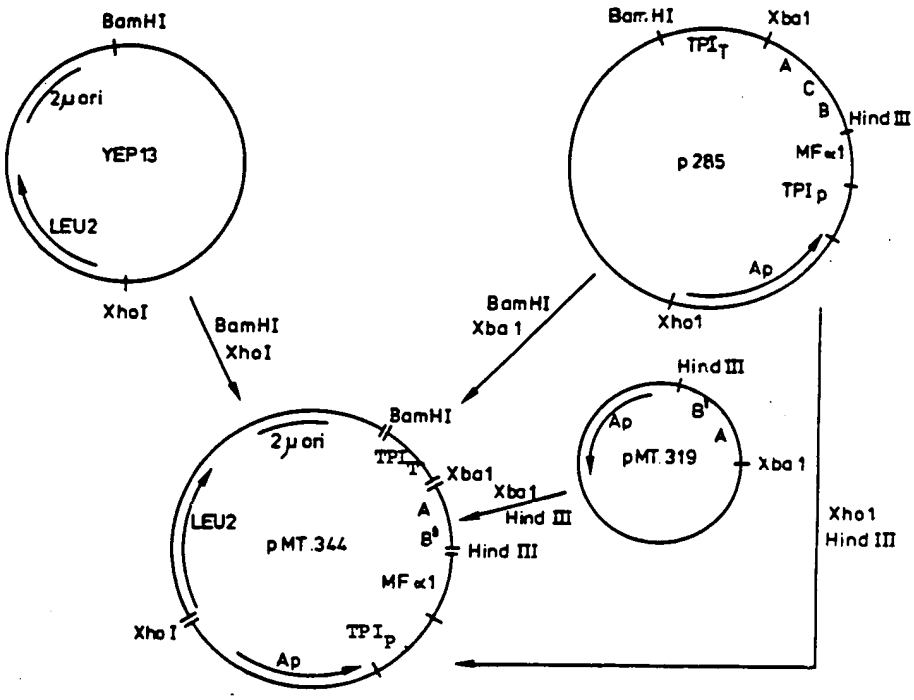
Assistant Examiner—Patricia Carson
Attorney, Agent, or Firm—Morris Fidelman; Franklin D. Wolffe

[57] **ABSTRACT**

Human insulin precursors containing the peptide chain B(1-29)–A(1-21) of human insulin and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)-chain are prepared by culturing a yeast host transformed with a replicable expression vehicle capable of expressing a DNA-sequence encoding the insulin precursor. The bridging chain is preferably relatively short and contains preferably from 2 to 8 amino acid residues. The bridging chain must not contain two adjacent basic amino acid residues (Lys or Arg) and has one Lys or Arg connected to the amino terminus of the A(1-21)-chain. Human insulin is prepared from the insulin precursors by in vitro conversion.

32 Claims, 8 Drawing Sheets

FIG. 1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TPI p
Ap
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Hind III
Ap
B'
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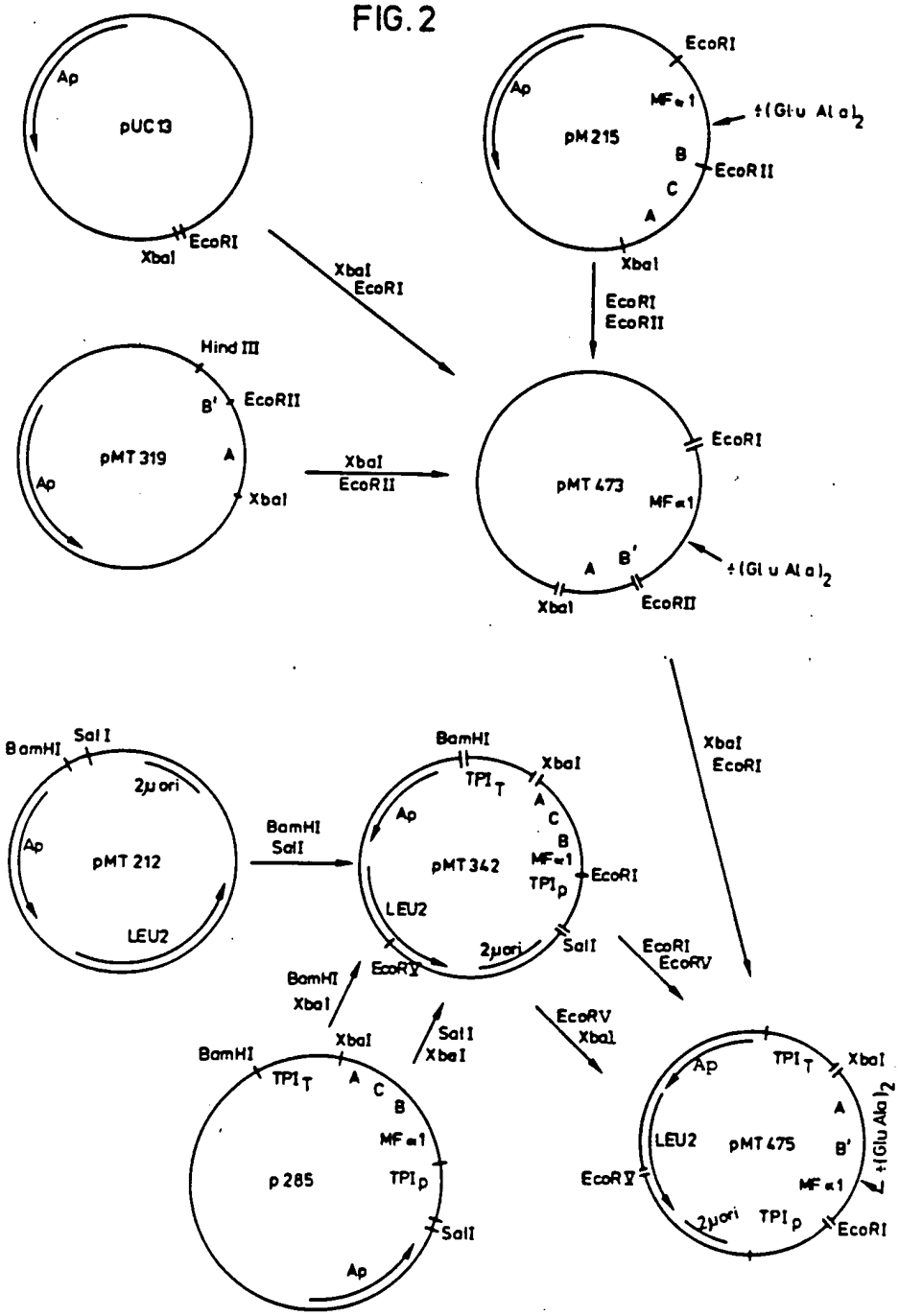
FIG. 2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Ap
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pMT473
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XbaI
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pMT212
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SalI
2μori
Ap
LEU2
BamHI
SalI
pMT342
BamHI
XbaI
TPIT
A
C
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MFα1
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Ap
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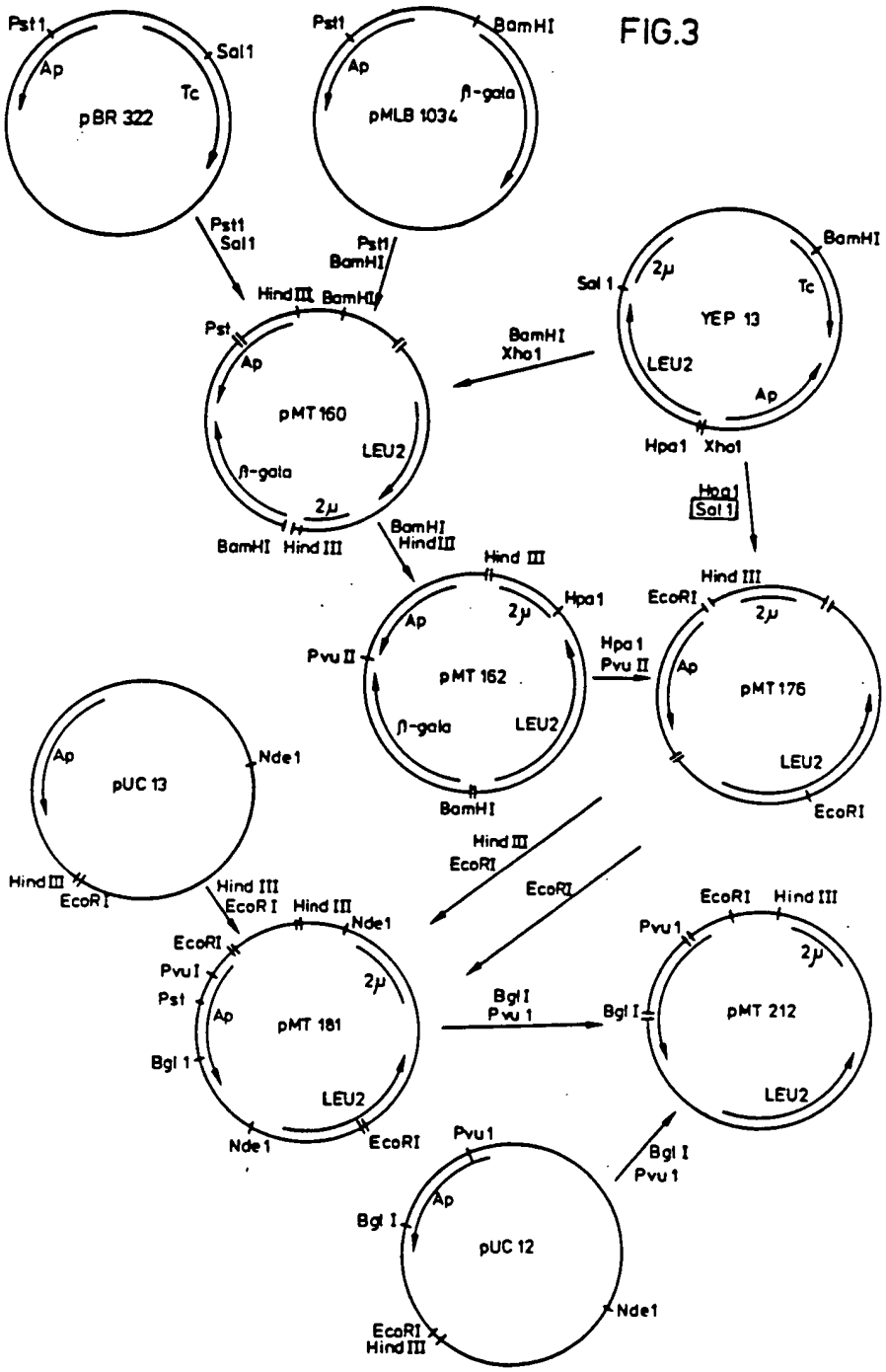
FIG.3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2μ
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Xho1
BamHI
Sal 1
2μ
Tc
YEP 13
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Sal 1
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Hind III
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Pvu II
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2μ
Ap
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Ap
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EcoR I
Hind III
EcoR I
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Nde1
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Pst
Ap
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Hind III
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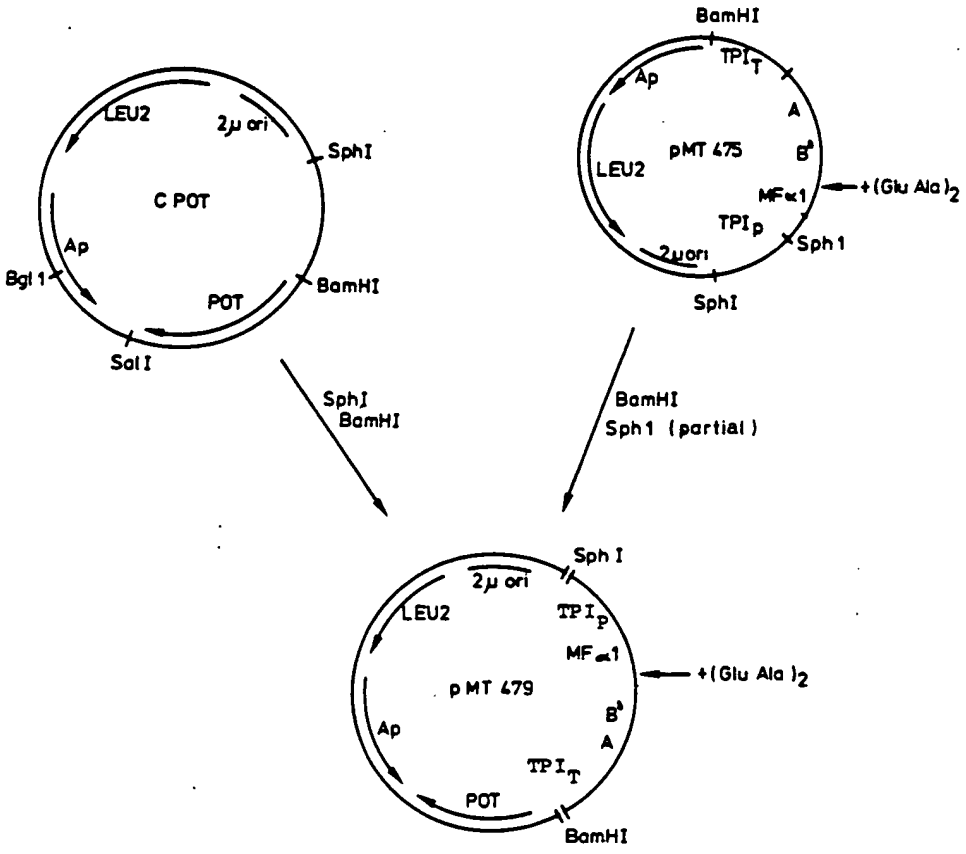
FIG. 4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Ap
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Ap
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MFα1
+(Glu Ala)2
TPI p
Sph 1
2μ ori
SphI
SphI
BamHI
BamHI
Sph1 (partial)
Sph I
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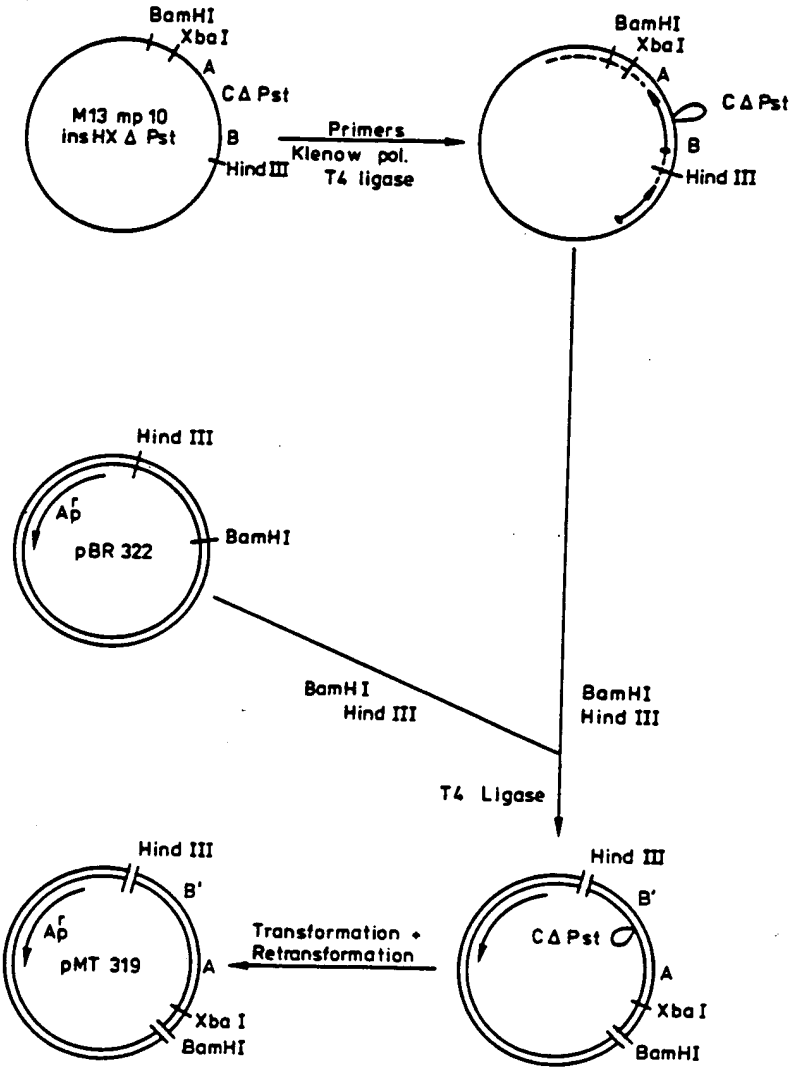
POT
BamHI

FIG. 5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C Δ Pst
M13 mp 10
ins HX Δ Pst
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Hind III
Primers
Klenow pol.
T4 ligase
BamHI
Xba I
A
C Δ Pst
B
Hind III
Hind III
Ap r
pBR 322
BamHI
BamHI
Hind III
BamHI
Hind III
T4 Ligase
Hind III
B'
Ap r
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A
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BamHI
Transformation +
Retransformation
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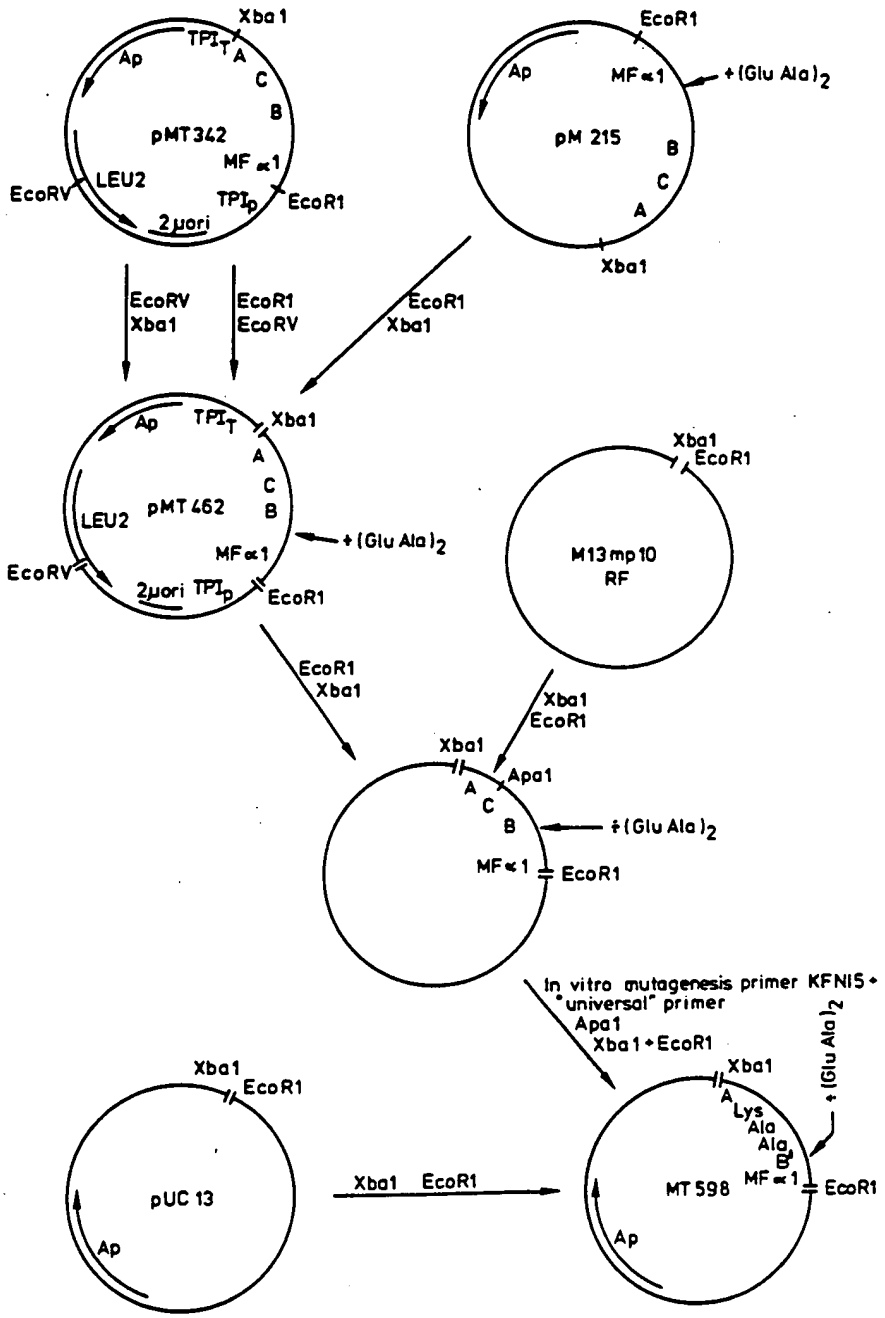
B'
C Δ Pst
A
Xba I
BamHI

FIG.6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Xba1
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A
C
B
+(Glu Ala)$_2$
MF ∝1
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"universal" primer
Apa1
Xba1+EcoR1
+(Glu Ala)$_2$
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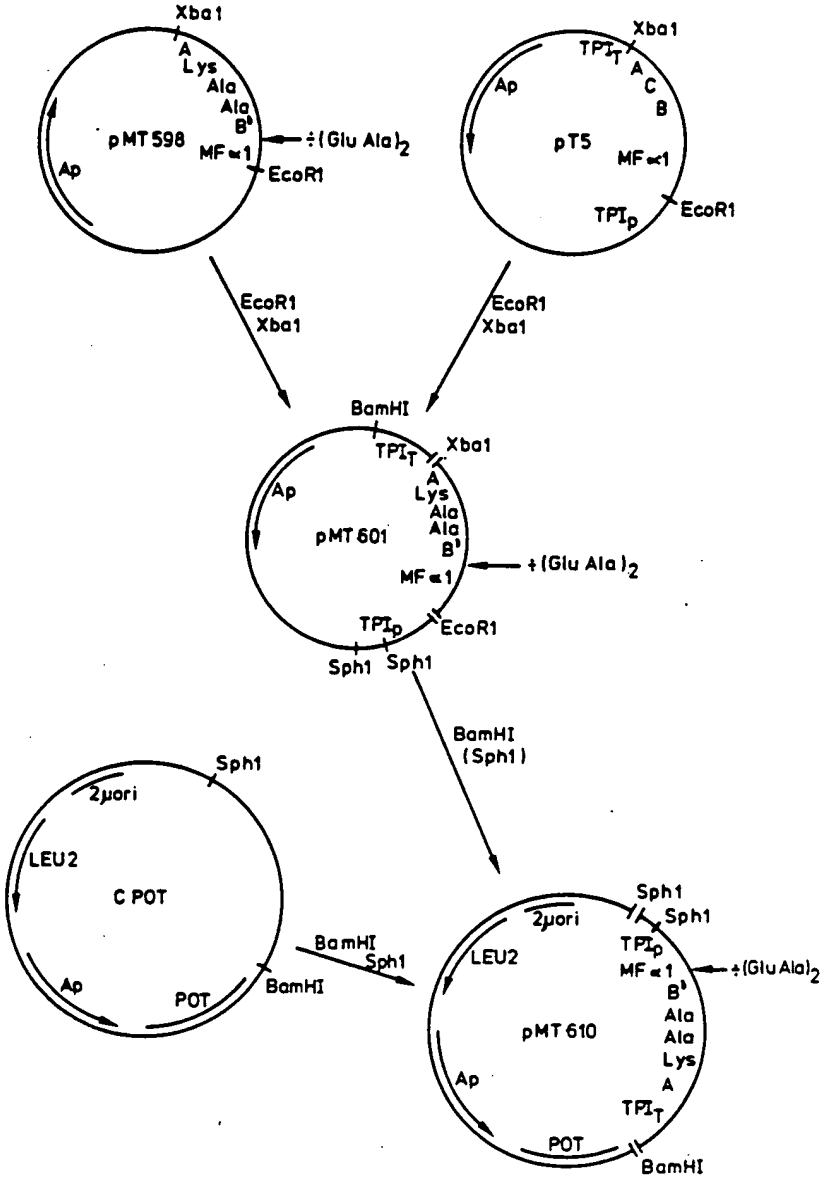
FIG. 7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C
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pT5
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EcoR1
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EcoR1
Xba1
BamHI
TPIT
Xba1
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Ala
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Sph1
Sph1
BamHI
(Sph1)
Sph1
2μori
LEU2
C POT
Ap
POT
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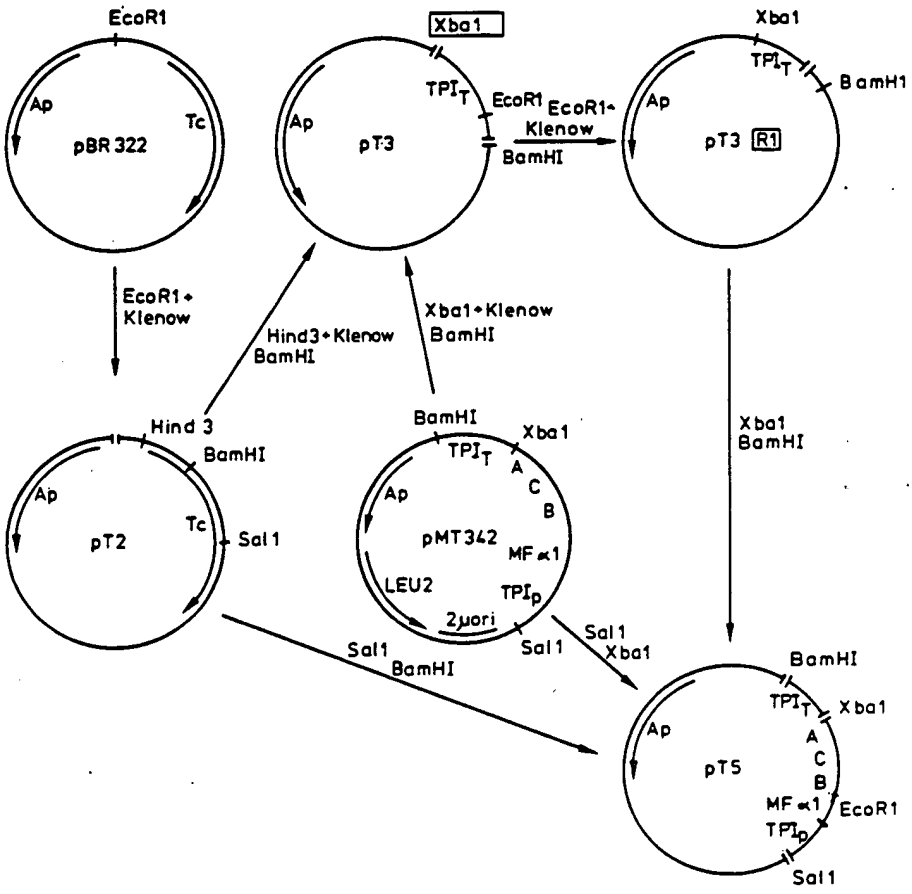
FIG. 8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Ap
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EcoR1+ Klenow
Xba1
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BamH1
Ap
pT3 R1
EcoR1+ Klenow
Hind3+Klenow BamHI
Xba1+Klenow BamHI
Xba1 BamHI
Hind 3
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Ap
Tc
pT2
Sal1
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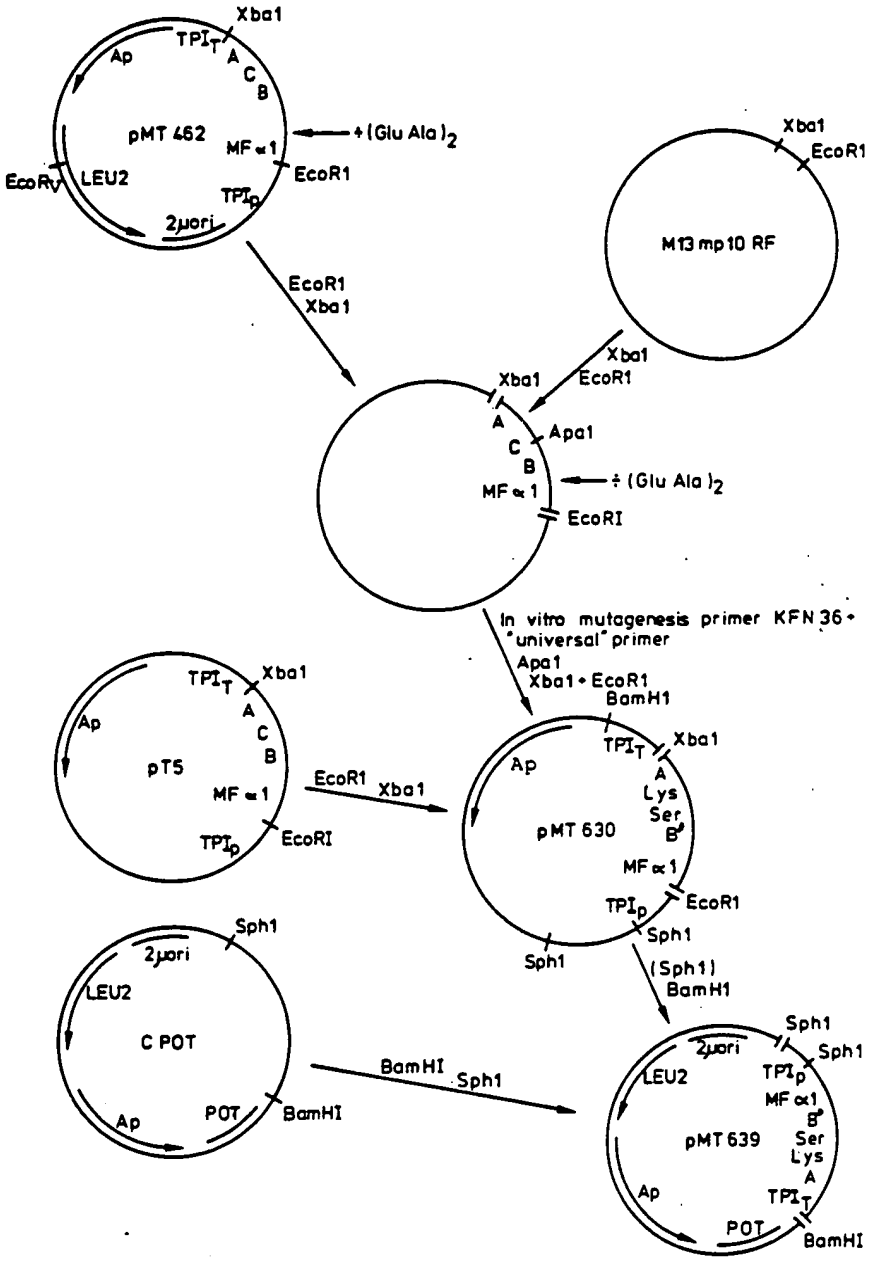
FIG. 9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2μori
EcoR1
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M13 mp10 RF
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EcoR1
A
Apa1
C
B
MF ∝1
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EcoRI
In vitro mutagenesis primer KFN 36 + "universal" primer
Apa1
Xba1 + EcoR1
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BamHI Sph1
Sph1
2μori
Sph1
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pMT 639
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POT
BamHI

DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

This invention relates to biosynthetic insulin. More specifically, the invention is directed to DNA-sequences encoding biosynthetic insulin precursors and to the preparation of such insulin precursors which are convertible into biosynthetic human insulin by in vitro conversion.

BACKGROUND OF THE INVENTION

In the past insulin has been synthezised (from synthetic A- and B-chains) or re-synthesized (from naturally derived A- and B-chains) by combining the two chains in an oxidation process whereby the 6 cysteine sulfhydryl groups of the reduced chains (4 in the A-chain, 2 in the B-chain) are converted into disulfide bonds. By this method disulfide bonds are formed largely at random, meaning that the yield of insulin with disulfide bridges correctly positioned between cysteine residues A-6 and A-11, A-7 and B-7, and A-20 and B-19, respectively, is very low.

Following the discovery of proinsulin as a biological precursor of insulin it was observed that the A- and B-polypeptide moieties of the linear-chain totally reduced proinsulin (those moieties corresponding to the A- and B-chains of insulin, respectively) could be oxidatively combined with much less randomization of the disulfide bonds to give a substantially higher yield of correctly folded proinsulin as compared with the combination of free A- and B-chains (D. F. Steiner et al.: Proc. Nat. Acad. Sci. 60 (1968), 622). Albeit high yields were obtained only at proinsulin concentrations too low to make the process feasible on a preparative scale, the function of the C- (i.e. connecting peptide) moiety of the B-C-A polypeptide sequence of proinsulin, namely that of bringing the 6 cysteine residues into spatial positions favorable for correct oxidation into proinsulin, was clearly demonstrated.

The proinsulin formed may function as an in vitro precursor of insulin in that the connecting peptide is removable by enzymatic means (W. Kemmler et al.: J. Biol. Chem. 246 (1971), 6786).

Subsequently it has been shown that proinsulin-like compounds having shorter linking moieties than the C-peptide and flanked at both ends by specific enzymatic or chemical cleavage sites (the so-called miniproinsulins (A. Wollmer et al., Hoppe-Seyler's Z. Physiol. Chem. 355 (1974), 1471–1476 and Dietrich Brandenburg et al., Hoppe-Seyler's Z. Physiol. Chem. 354 (1973), 1521–1524)) may also serve as insulin precursors.

Endeavours to provide biosynthetic insulins, particularly that identical to the human species, have followed the same strategic pathways as those to synthetic insulin. The insulin A- and B-chains have been expressed in separate host organisms, isolated therefrom and then combined as described supra (R. E. Chance et al.: Diabetes Care 4 (1982), 147). Microorganisms have been transformed with cloning vectors encoding preproinsulin or proinsulin which may be secreted as such (W. Gilbert et al.: European Patent Publ. No. 6694) or accumulated intracellularly as hybrid gene products (D. V. Goeddel et al.: European Patent Publ. No. 55945). The miniproinsulin pathway has also been attempted (D. V. Goeddel, supra).

Procuring the A- and B-chains in separate fermentation processes followed by combination of the chains is inherently impractical. The dual fermentation inconvenience may be overcome by choosing the proinsulin or miniproinsulin strategy. However, the use of a proinsulin as the biosynthetic insulin precursor may entail certain disadvantages. The proinsulin, whether excreted into the fermentation liquid as such or accumulated intracellularly in the host organism, possibly as a hybrid gene product, is likely to contain substantially randomized disulfide bonds. The refolding of such "scrambled" products into correctly folded proinsulin may be conducted either directly (H.—G. Gattner et al.: Danish Patent Application No. 4523/83) or via the single chain hexa-S-sulfonate (F. B. Hill: European Patent Publ. No. 37255). The refolding process usually entails some degree of polymerization and hence the inconvenience of using laborious purification steps during recovery.

In addition, insulin precursors of the proinsulin type are prone to undergo enzymatic degradation, either within the host cells or following its excretion into the fermentation broth. In yeast it has been shown that human proinsulin is particularly sensitive to enzymatic cleavages at the two dibasic sequences (Arg31–Arg32 and Lys64–Arg65). Apparently these cleavages occur before the establishment of the S—S bridges, resulting in the formation of C-peptide, A-chain and B-chain.

OBJECT OF THE INVENTION AND SUMMARY THEREOF

The object of the present invention is to circumvent these disadvantages by devising biosynthetic insulin precursors which are generated largely with correctly positioned disulfide bridges between the A- and B-moieties and, furthermore, substantially more resistant to proteolytic degradation than the biosynthetic insulin precursors known heretofore.

A single chain insulin precursor consisting of a shortened insulin B-chain from Phe^{B1} to Lys^{B29} continuing into a complete A-chain from Gly^{A1} to Asn^{A21}, B(1-29)-A(1-21), is known (Jan Markussen, "Proteolytic degradation of proinsulin and of the intermediate forms",: Proceedings of the Symposium on Proinsulin, Insulin and C-Peptide, Tokushima, 12–14 July, 1978, Editors: S. Baba et al.). This insulin precursor B(1-29)-A(1-21) is prepared by a semisynthetic process from porcine insulin. First the insulin B(1-29) and A(1-21) chains were prepared and coupled to form a linear peptide B(1-29)-A(1-21). This compound in the hexathiol form was oxidized in vitro rendering the single chain des-(B30) insulin molecule.

The present invention is based on the surprising discovery that the above single chain insulin precursor B(1-29)-A(1-21) and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)-chain are expressed in high yields and with correctly positioned disulfide bridges when culturing yeast strains transformed with DNA-sequences encoding such insulin precursors are cultured.

According to a first aspect of the present invention there is provided a DNA-sequence encoding insulin precursors of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21) \qquad I$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n is an integer from 0 to 33, m is 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) is the A chain of human insulin, with the proviso that the peptide chain $-X_n-Y-$ does not contain two adjacent basic amino acid residues (i.e. Lys and Arg).

Preferred insulin precursors of the above formula I are B(1-29)-A(1-21), i.e. m=0 in formula I, and compounds with a relative short bridging chain between the B(1-29)- and the A(1-21)-chain.

When m=1, then n is preferably 1-33, more preferably 1-15, 1-8 or 1-5 and most preferably 1-3 or 1-2. X may preferably be selected from the group consisting of Ala, Ser and Thr, the individual X's being equal or different. Examples of such preferred compounds are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a second aspect of the present invention there is provided a replicable expression vehicle capable of expression of a DNA-sequence comprising a sequence encoding the insulin precursors of formula I in yeast.

The expression vehicle may be a plasmid capable of replication in the host microorganism or capable of integration into the host organism chromosome. The vehicle employed may code for expression of repeated sequence of the desired DNA-sequence, each separated by selective cleavage sites.

According to a third aspect of the present invention there is provided a process for producing insulin precursors of formula I in yeast wherein a transformant yeast strain including at least one expression vehicle capable of expressing the insulin precursors is cultured in a suitable nutrient medium followed by isolation of the insulin precursors.

According to a fourth aspect of the present invention there are provided novel human insulin precursors. Such novel human insulin precursors have the following general formula

$$B(1\text{-}29)\text{-}X_n\text{-}Y\text{-}A(1\text{-}21) \qquad II$$

in which the different symbols have the above mentioned definitions. Preferred novel insulin precursors are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a fifth aspect of the present invention there is provided a yeast strain transformed with an expression vehicle capable of expressing a DNA-sequence comprising a sequence encoding the above insulin precursors in yeast.

The insulin precursors may be expressed with additional protein proceeding the insulin precursor. The additional protein may have the function of protecting the insulin precursor against, e.g. in vivo degradation by endogeneous enzymes or of providing information necessary to transport the desired protein into the periplasmic space and finally across the cell wall into the medium.

The additional protein contains a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors enabling subsequent splitting off of the additional protein either by the microorganism itself or by later enzymatical or chemical cleavage.

Accordingly the present invention includes a DNA-sequence encoding the above insulin precursors and further comprising an additional DNA-sequence positioned upstream to the sequence encoding the insulin precursors and encoding an additional amino acid-sequence containing a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors.

According to a preferred embodiment of the present invention the additional amino acid sequence comprises at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor.

When the insulin precursor is expressed in yeast the additional amino acid-sequence may contain two basic amino acids (e.g. Lys-Lys, Arg-Arg, Lys-Arg or Arg-Lys) adjacent to N-terminal of the B(1-29)-chain of the insulin precursor, yeast being able to cleave the peptide bond between the basic amino acids and the precursor. Also a Glu-Ala or Asp-Ala cleavage site adjacent to the desired protein enables separation of the additional amino acid sequence by the yeast itself by means of a dipeptidase enzyme produced by the yeast.

The insulin precursors may be secreted with an amino acid-sequence linked to the B(1-29)-chain of the precursors provided that this amino acid sequence contains a selective cleavage site adjacent to the B(1-29)-chain for later splitting of the superfluous amino acid sequence. If the insulin precursors do not contain methionine cyanogen bromide cleavage at methionine adjacent to the desired protein would be operative. Likewise, arginine- and lysine-cleavage sites adjacent to the desired protein enables cleavage with trypsinlike proteases.

For secretion purposes the DNA-sequence encoding the insulin precursors may be fused to an additional DNA-sequence coding for a signal peptide. The signal peptide is cleaved off by the transformant microorganism during the secretion of the expressed protein product from the cells ensuring a more simple isolation of the desired product. The secreted product may be the insulin precursor or may contain an additional N-terminal amino acid-sequence to be removed later as explained above.

Secretion may be provided by including in the expression vehicle the yeast MFα1 leader sequence (Kurjan, J. and Herskowitz, I., Cell 30, (1982), 933–943) and according to a further preferred embodiment of the present invention the additional amino acid-sequence positioned upstream to the sequence encoding the insulin precursors comprises the yeast MFα1 leader coding sequence or part thereof.

The expression of the desired DNA-sequence will be under control of a promoter sequence correctly positioned to the DNA-sequence encoding the desired protein product to result in expression of the desired protein in the host organism. Preferably a promoter from a gene indigenous to the host organism may be used. The DNA-sequence for the desired protein will be followed by a transcription terminator sequence, preferably a terminator sequence from a gene indigenous to the host organism. If yeast is used as host organism the promoter and terminator sequences are preferably the promoter and terminator of the triose phosphase isomerase (TPI) gene, respectively.

Other promoters may be utilized such as the phosphoglycerate kinase (PGK1)- and the MFα1-promoter.

The present invention further comprises a method for preparing human insulin by which a yeast strain is transformed with a replicable expression vehicle comprising a DNA-sequence encoding the insulin precursors of the above formula I, the transformed yeast strain is cultured in a suitable nutrient medium, the insulin precursors are

recovered from the culture medium and converted in vitro into human insulin.

The insulin precursors according to the present invention may be converted into mature human insulin by transpeptidation with an L-threonine ester in the presence of trypsin or a trypsin derivative as described in the specification of U.S. Pat. No. 4,343,898 (the disclosure of which is incorporated by reference hereinto) followed by transformation of the threonine ester of human insulin into human insulin by known processes.

If the insulin precursors are secreted with an additional amino acid sequence adjacent to the N-terminal of the B(1-29)-chain such amino acid sequence should either be removed in vitro before the transpeptidation or should contain at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain as trypsin will cleave the peptide bond between the basic amino acid and the amino group of Phe^{B1} during the transpeptidation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate a preferred embodiment of the present invention.

FIG. 1 illustrates the preparation of plasmid pMT344,
FIG. 2 illustrates the preparation of plasmid pMT475,
FIG. 3 illustrates the preparation of plasmid pMT212,
FIG. 4 illustrates the preparation of plasmid pMT479
FIG. 5 illustrates the preparation of plasmid pMT319,
FIG. 6 illustrates the preparation of plasmid pMT598,
FIG. 7 illustrates the preparation of plasmid pMT610,
FIG. 8 illustrates the preparation of plasmid pT5, and
FIG. 9 illustrates the preparation of plasmid pMT639.

In the drawings and part of the following description the expression B' is used instead of B(1-29) and A instead of A(1-21). Accordingly the expression B'A is equivalent to the expression B(1-29)-A(1-21).

DETAILED DESCRIPTION

1. Preparation of a gene coding for human proinsulin B-C-A

Total RNA purified (Chirgwin, J. M. Przybyla, A. E., McDonald, R. J. & Rutter, W. J., Biochemistry 18, (1979) 5294–5299) from human pancreas was reverse transcribed (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J. F. & Marcker, K. A., Proc. Natl. Acad. Sci. USA 80, (1983), 2866–2869) with AMV reverse transcriptase and d(GCTTTATTCCATCTCTC) as 1. strand primer. After preparative urea-polyacrylamide gel purification of the human proinsulin cDNA, the second strand was synthesized on this template with DNA polymerase large fragment and d(CAGATCACTGTCC) as 2nd strand primer. After S1 nuclease digestion the human proinsulin ds. cDNA was purified by polyacrylamide gel electrophoresis, tailed with terminal transferase and cloned in the PstI site on pBR327 (Sorberon et al., Gene 9, (1980), 287–305) in *E. coli*. A correct clone harbouring a plasmid containing a gene encoding human proinsulin B-C-A was identified from the recombinants by restriction endonuclease analysis and confirmed by nucleotide sequencing (Maxam, A., & Gilbert, W., Methods in Enzymology, 65 (1980), 499–560. Sanger, F., Nicklen, S. and Coulson, A. R., Proc. Natl. Acad. Sci. USA 74, (1977), 5463–5467).

2. Preparation of genes coding for precursors of human insulin

The gene encoding B(1-29)-A(1-21) of human insulin was made by site specific mutagenesis of the human proinsulin sequence with a 75 bp in frame deletion in the C-peptide coding region inserted into a circular single stranded M-13 bacteriophage vector. A modified procedure (K. Norris et al., Nucl. Acids. Res. 11 (1983) 5103–5112) was used in which a chemically synthesized 19-mer deletion primer was annealed to the M13 template. After a short enzymatic extension reaction a "universal" 15-mer M13 dideoxy sequencing primer was added followed by enzymatic extension and ligation. A double stranded restriction fragment (BamHI-Hind III) was cut out of the partly double stranded circular DNA and ligated into pBR322 cut with BamHI and Hind III.

The obtained ligation mixture was used to transform *E. coli* and transformants harbouring a plasmid pMT319 containing the gene encoding B(1-29)-A(1-21) of human insulin were identified.

Genes encoding B(1-29)-Ala-Ala-Lys-A(1-21) and B(1-29)-Ser-Lys-A(1-21) were made accordingly by insertion of a fragment encoding MFα1-B-C-A in the M-13 bacteriophage and site specific mutagenesis of the human proinsulin sequence with chemically synthesized 30-mer and 27-mer deletion primers, respectively, and the above mentioned "universal" 15-mer M13 dideoxy sequencing primer. A double stranded restriction fragment (XbaI-EcoRI) was cut out of the partly double stranded circular DNA and ligated into pUC13 and pT5, respectively. By transformation and retransformation of *E. coli*, transformants harbouring a plasmid pMT598 containing the gene encoding B(1-29)-Ala-Ala-Lys-A(1-21) and pMT630 containing the gene encoding B(1-29)-Ser-Lys-A(1-21) were identified.

A gene encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was made in a similar way as described above by insertion of a fragment encoding MFα1-B(1-29)-A(1-21) in a M13 mp11 bacteriophage and site specific mutagenesis of the B(1-29)-A(1-21) sequence with a chemically synthesized 46-mer deletion primer (5'-CACACCCAAGACTAAAGAAGCTGAAGACTTGCAAAGAGGCATTGTG-3') and the "universal" primer. Also, by a similar procedure a gene encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-A(1-21) was constructed.

3. Plasmid constructions

The gene encoding B(1-29)-A(1-21) of human insulin (B'A) was isolated as a restriction fragment from pMT319 and combined with fragments coding for the TPI promoter (TPI_P) (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of *Saccharomyces cerevisiae*. J. Mol. Applied Genet. 1 (1982) 419–434), the MFα1 leader sequence (J. Kurjan and I. Herskowitz,. Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933–943) and the transcription termination sequence from TPI of *S. cerevisiae* (TPI_T). These fragments provide sequences to ensure a high rate of transcription for the B'A encoding gene and also provide a presequence which can effect the localization of B'A into the secretory pathway and its eventual excretion into the growth medium. This expression unit for B'A

(TPIρ-MFα1 leader—B'A—TPIγ was then placed on a plasmid vector containing the yeast 2μ origin of replication and a selectable marker, LEU 2, to give pMT344, a yeast expression vector for B'A.

During in vivo maturation of α-factor in yeast, the last (C-terminal) six amino acids of the MFα1 leader peptide (Lys-Arg-Glu-Ala-Glu-Ala) are removed from the α-factor precursor by the sequential action of an endopeptidase recognizing the Lys-Arg sequence and an aminodipeptidase which removes the Glu-Ala residues (Julius, D. et al. Cell 32 (1983) 839–852). To eliminate the need for the yeast aminodipeptidase, the sequence coding for the C-terminal Glu-Ala-Glu-Ala of the MFα1 leader was removed via in vitro mutagenesis. The resulting yeast expression plasmid, pMT475, contains the insert coding for TPIρ-MFα1 leader (minus Glu-Ala-Glu-Ala)—B'A—TPIγ.

In a preferred construction the modified expression unit was transferred to a stable, high copy number yeast plasmid CPOT, (ATCC No. 39685), which can be selected merely by the presence of glucose in the growth medium. The resulting yeast expression vector for B'A was numbered pMT479.

The fragment encoding MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21) was isolated as a restriction fragment from pMT598 and combined with fragments coding for the TPI promoter and the TPI terminator and transferred to the above mentioned high copy number yeast plasmid CPOT. The resulting yeast expression vector for B(1-29)-Ala-Ala-Lys-A(1-21) was numbered pMT610.

The fragment containing the insert TPIρ-MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPIγ was isolated as a restriction fragment from pMT630 and transferred into CPOT. The resulting yeast expression vector for B(1-29)-Ser-Lys-A(1-21) was numbered pMT639.

The fragment containing the insert TPIρ-MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21)-TPIγ was inserted into a high copy number yeast plasmid DPOT, being a CPOT derivative containing a SphI-BamHI-fragment of pBR322 inserted into a SpHI-BamHI fragment of CPOT. The resulting yeast expression vector for B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was numbered p1126.

4. Transformation

Plasmids pMT344 and pMT475 were transformed into *S. cerevisiae* leu 2 mutants by selection for leucin prototrophy as described by Hinnen et al. (A. Hinnen, J. B. Hicks and G. R. Fink. Transformation of Yeast. Proc. Nat. Aca. Sci. 75 (1978) 1929).

Plasmids pMT479, pMT610, pMT639 and p1126 were transformed into *S. cerevisiae* strains carrying deletions in the TPI gene by selecting for growth on glucose. Such strains are normally unable to grow on glucose as the sole carbon source and grow very slowly on galactose lactate medium. This defect is due to a mutation in the triose phosphate isomerase gene, obtained by deletion and replacement of a major part of this gene with the *S. cerevisiae* LEU 2 gene. Because of the growth deficiencies there is a strong selection for a plasmid which contains a gene coding for TPI. pMT479 contains the *Schizo. pombe* TPI gene.

5. Expression of human insulin precursors in yeast

Expression products of human insulin type were measured by radioimmunoassay for insulin as described by Heding, L. (Diabetologia 8, 260–66, 1972) with the only exception that the insulin precursor standard in question was used instead of an insulin standard. The purity of the standards were about 98% as determined by HPLC and the actual concentration of peptide in the standard was determined by amino acid analysis. The expression levels of immunoreactive human insulin precursors in the transformed yeast strains are summarized in Table 1.

TABLE 1

Expression levels of immunoreactive human insulin precursors in yeast.

Yeast strain	Plasmid	Construct	Immunoreactive insulin precursor (nmol/l supernatant)
MT 350 (DSM 2957)	pMT 344	B(1-29)-A(1-21)	100
MT 371 (DSM 2958)	pMT 475	B(1-29)-A(1-21)	192
MT 519 (DSM 2959)	pMT 479	B(1-29)-A(1-21)	2900
MT 620 (DSM 3196)	pMT 610	B(1-29)-Ala—Ala—Lys-A(1-21)	1200–1600
MT 649 (DSM 3197)	pMT 639	B(1-29)-Ser—Lys-A(1-21)	1600
ZA 426	p1126	B(1-29)-Thr—Arg—Glu—Ala—Glu—Asp—Leu—Gln—Lys-A(1-21)	200

The isolation and characterization of expression products are given in Examples 7–9 and 12–13.

6. Conversion of human insulin precursor into B30 esters of human insulin

The conversion of the human insulin precursors into human insulin esters can be followed quantitatively by HPLC (high pressure liquid chromatography) on reverse phase. A 4×300 mm "μBondapak C18 column" (Waters Ass.) was used and the elution was performed with a buffer comprising 0.2M ammonium sulphate (adjusted to a pH value of 3.5 with sulphuric acid) and containing 26–50% acetonitrile. The optimal acetonitrile concentration depends on which ester one desires to separate from the insulin precursor. In case of human insulin methyl ester separation is achieved in about 26% (v/v) of acetonitrile.

Before the application on the HPLC column the proteins in the reaction mixture were precipitated by addition of 10 volumes of acetone. The precipitate was isolated by centrifugation, dried in vacuo, and dissolved in 1M acetic acid.

EXPERIMENTAL PART

Example 1

Construction of a gene coding for B(1-29)-A(1-21)insulin

Materials and Methods

"Universal" 15-mer M13 dideoxy sequencing primer d(TCCCAGTCACGACGT), T4 DNA ligase and restriction enzymes were obtained from New England Biolabs. DNA polymerase I "Klenow fragment" and

T_4 polynucleotide kinase were purchased from P-L Biochemicals. (γ-^{32}P)-ATP (7500 Ci/mmol) was obtained from New England Nuclear. The support for oligonucleotide synthesis was 5'-O-dimethoxytrityl N^2-isobutyryldeoxyguanosine bound via a 3'-O-succinyl group to aminomethylated 1% crosslinked polystyrene beads from Bachem.

Construction of M13 mp10 insHX PstΔphage:

The M13 mp10 derived phage mp10 insHX was constructed by cloning of the 284 bp large proinsulin coding Hind III-XbaI fragment, isolated from p285, into Hind III-XbaI cut M13 mp10 RF.M13 mp10 RF is available from P-L Biochemicals, Inc. Milwaukee, Wis. (Catalogue No. 1541).

M13 mp10 insHXΔPst was constructed from mp10 insHX,RF by complete PstI digestion followed by ligation and transformation of *E. coli* JM103. The resulting phage harbours the human proinsulin coding sequences, with a 75 bp in frame deletion in the C-peptide coding region. Single stranded phage was prepared as described (Messing, J. and Vieira, J. (1982) Gene 19, 269–276).

Oligodeoxyribonucleotide synthesis

The 19-mer deletion primer d(CACACCCAAGGGCATTGTG) was synthesized by the triester method on a 1% crosslinked polystyrene support (Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) Nucl. Acids Res. 10, 1755–1769). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an HPLC pump and a control module. The oligonucleotide was purified after deprotection by HPLC on a LiChrosorb RP18 column (Chrompack (Fritz, H.-J., Belagaje, R., Brown, E. L., Fritz, R. H., Jones, R. A., Lees, R. G., and Khorana, H. G. (1978) Biochemistry 17, 1257–1267).

5'-^{32}P-labelling of oligodeoxyribonucleotide

The 19-mer was labelled at the 5'end in a 60 μl reaction mixture containing 50 mM Tris-HCl at pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 0.4% glycerol, 120 pmole ATP, 50 μCi of (γ-^{32}P)-ATP (10 pmole), 120 pmole of oligonucleotide and 30 units of T4 polynucleotide kinase. The reaction was carried out at 37° C. for 30 min., and terminated by heating at 100° C. for 3 min. The labelled oligonucleotide was separated from unreacted (γ-^{32}P)-ATP by chromatography on a column (1×8 cm) of Sephadex G50 superfine in 0.05M triethylammonium bicarbonate at pH 7.5.

For colony hybridization the oligonucleotide was labelled without the addition of "cold" ATP as described (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J., and Marcker, K. (1983) Proc. Natl. Acad. Sci. USA 80, 2866–2869).

Oligodeoxyribonucleotide primed DNA synthesis

Single stranded M13 mp10 insHXΔPst (0.4 pmole was incubated with the 19-mer 5'-(^{32}P)-labelled oligodeoxyribonucleotide primer (10 pmole) in 20 μl of 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DDT for 5 min. at 55° C. and annealed for 30 min. at 11° C. Then 9 μl of d-NTP-mix consisting of 2.2 mM of each dATP, dCTP, dGTP, dTTP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DDT was added followed by 7 units of *E. coli* DNA polymerase I (Klenow). The mixture was kept for 30 min. at 11° C. and heated for 10 min. at 65° C. 15-mer universal primer for dideoxy sequencing (4 pmole) was added and the mixture heated at 65° C. for an additional minute. After cooling to 11° C. 26 μl of solution containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.8 mM of each dATP, dCTP, dGTP, dTTP, 2.4 mM ATP and 10^3 units of T4 ligase was added followed by 9.5 units of *E. coli* DNA polymerase I (Klenow). The final volume of the mixture was 64 μl. After incubation for 3 hours at 11° C. 20 μl 4M sodium acetate was added, and the volume adjusted to 200 μl with TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The mixture was extracted twice with phenol/-chloroform. 0.9 μg (0.3 pmole) of the purified large fragment of pBR322 cleaved with BamHI and Hind III was added as carrier DNA. After ether extraction of the aqueous phase, the DNA was isolated by ethanol precipitation.

Endonuclease digestion

The DNA, prepared as described above, was digested respectively with 16 and 20 units of restriction endonucleases BamHI and Hind III in a total volume of 22 μl of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DDT, 4 mM spermidine). The mixture was extracted with phenol/chloroform followed by ether and the DNA was isolated by ethanol precipitation and then dissolved in 12 μl H_2O. 2 μl was used for electrophoresis on a 7M urea 6% polyacrylamide gel.

Ligation

To a part of the DNA (5 μl) was added a new portion of the purified large fragment of pBR322 cut with BamHI and Hind III (0.38 μg) and 400 units of T4 DNA ligase, in a total volume of 41 μl containing 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DDT, 40 μg/ml gelatine. Ligation was performed at 16° C. for 16 hours.

Transformation

20.5 μl of the ligation mixture was used to transform $CaCl_2$ treated *E. coli* MC 1000 (r^-, m^+). The bacteria were plated on LB-agar plates and selected for resistance to ampicillin (100 μg/ml). 2.6×10^3 colonies per pmole of M13 mp10 insHXΔPst were obtained.

Colony hybridization

123 transformed colonies were picked onto fresh ampicillin plates and grown overnight at 37° C. Colonies were transferred to Whatman 540 filter paper and fixed (Gergen, J. P., Stern, R. H., and Wensink, P. C. (1979), Nucl. Acids Res. 7, 2115–2136). A prehybridization was performed in a sealed plastic bag with 6 ml of 0.9M NaCl, 0.09M Tris-HCl pH 7.5 0.006M EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1% SDS and 50 μg/ml salmon sperm DNA for 2 hours at 65° C. Then 8.5×10^6 cpm of ^{32}P-labelled 19-mer was added and hybridization performed at 45° C. overnight. The filter was washed with 0.9M NaCl, 0.09M sodium citrate three times at 0° C. for 5 min. and was then autoradiographed and washed once at 45° C. for 1 min. and autoradiographed again. After washing at 45° C., identification of 3 colonies containing mutated plasmid was possible.

Endonuclease analysis of mutated plasmids

Plasmids from the supposed mutant colonies were prepared by a rapid method (Ish-Horowicz, D. and

Burke, J. F. (1981), Nucl. Acids Res. 9, 2989–2998), digested with a mixture of BamHI and Hind III and then analysed by electrophoresis on a 2% agarose gel. The presence of a 179 bp fragment confirmed that the 3 colonies contained mutant plasmid.

Retransformation

The colonies identified as "mutant" contain plasmids which are the progeny of a heteroduplex. Pure mutant could be obtained by retransformation of $CaCl_2$ treated *E. Coli* MC1000 (r^-, m^+) with plasmid from 2 of the mutant colonies. From each plate 5 ampicillin resistant clones were isolated, plasmid DNA was prepared and analysed by endonuclease cleavage as mentioned above. 3 out of 5 and 5 out of 5 respectively were shown to be pure mutant. One plasmid pMT319 was selected for further use.

DNA sequence analysis

5 μg of pMT319 was cleaved with BamHI under standard conditions, phenol extracted and ethanol precipitated. Filling in of the BamHI sticky ends was performed with Klenow DNA polymerase I, dCTP, dGTP, dTTP, and α-^{32}P-dATP.

After phenol extraction and ethanol precipitation the DNA was digested with EcoRI. The ^{32}-P labelled fragment with the deletion was purified by electrophoresis on a 2% agarose gel and sequenced by the Maxam-Gilbert method (Maxam, A. and Gilbert, W. (1980) Methods in Enzymology 65, 499–560).

EXAMPLE 2

Construction of a yeast plasmid pMT344 for expression of B(1-29)-A(1-21) of human insulin (B'A)

Plasmid pMT319 containing the gene coding for B'A and constructed as explained above was cut with restriction enzymes Hind III and XbaI and a 0.18 kb fragment was isolated (T. Maniatis, E. F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) from a 2% agarose gel. Similarly a fragment (6.5 kb XhoI-Hind III) containing the *S. cerevisiae* TPI promotor (TPI_P) (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of *Saccharomyces cerevisiae*, J. Mol. Applied Genet. 1 (1982) 419–434) and the MFα1 leader sequence (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933–943) was isolated from plasmid p285 constructed as described in U.S.-patent application Ser. No. 547,748 of Nov. 1, 1983. P285 contains the insert TPI_P-MFα1 leader -B-C-A- TPI_T and was deposited in yeast strain Z33 (ATCC No. 20681). A fragment (0.7 kb XbaI-BamHI) containing the TPI transciption termination sequences (TPI_T) (T. Alber and G. Kawasaki, Nucleotide Sequence of the Triose Phosphate Isomerase Gene of *Saccharomyces cerevisiae*. J. Mol. Applied Genet. 1 (1982) 419–434) was also isolated from p285. Finally a 5.4 kb XhoI-BamHI fragment was isolated from the yeast vector YEp13 (J. R. Broach. Construction of High Copy Yeast Vectors Using 2 μm Circle Sequences. Methods Enzymology 101 (1983) 307–325). The above four fragments were ligated (T. Maniatis, E. F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) and transformed into *E. coli* (T. Maniatis, E. F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) selecting for ampicillin resistance. Plasmids were isolated from the transformants and the structure of one of these, pMT344, verified by restriction mapping. The construction and main features of pMT344 are outlined in FIG. 1.

EXAMPLE 3

Construction of a yeast plasmid pMT475 for expression of B(1-29)-A(1-21) of human insulin (B'A) after a modified MFα1 leader

To construct a plasmid for the expression of B'A after a MFα1 leader (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933–943) lacking its last four amino acids (Glu-Ala-Glu-Ala), the 0.14 kb XbaI-EcoRII fragment containing the A and part of the B' sequences was isolated from pMT319. Likewise the 5' proximal part of the B' gene was isolated as a 0.36 kb EcoRI-EcoRII fragment from pM215. Plasmid pM215 was constructed by subcloning the EcoRI-XbaI fragment containing the proinsulin B-C-A gene from p285 into pUC13 (constructed as described for pUC8 and pUC9 by Vieira et al., Gene 19: 259–268 (1982)) and subsequent in vitro loopout removal of the 12 bases coding for Glu-Ala-Glu-Ala at the junction between MFα1 leader and proinsulin B-C-A gene. These two pieces covering the B'A gene were ligated to EcoRI-XbaI digested pUC13 vector (see FIG. 2) to give pMT473. The modified gene contained within a 0.5 kb EcoRI-XbaI fragment was isolated from pMT473 and then ligated to two fragments (4.3 kb XbaI-EcoRV and 3.3 kb EcoRV-EcoRI) from pMT342, pMT342 is the yeast vector pMT212 with an inserted TPI_P-MFα1 leader -B-C-A- TPI_T. The resulting plasmid, pMT475, contains the insert: TPI_P-MFα1 leader (minus Glu-Ala-Glu-Ala) -B'A-TPI_T. The construction of plasmids pMT342, pMT473 and pMT475 is outlined in FIG. 2. The construction of the vector pMT212 is shown in FIG. 3. Plasmid pMLB1034 is described by M. L. Berman et al., Advanced Bacterial Genetics, Cold Spring Harbor (1982), 49–51 and pUC12 was constructed as described for pUC13 (Vieira et al, ibid.).

EXAMPLE 4

Insertion of the B(1-29)-A(1-21) (B'A) gene into a stable yeast plasmid pMT479

The modified B'A gene from pMT475 was isolated as a 2.1 kb BamHI-partial SphI fragment and ligated to an approximately 11 kb BamHI-SphI fragment of plasmid CPOT (ATCC No. 39685) to give plasmid pMT479 (FIG. 4). Plasmid CPOT is based on the vector C1/1 which has been modified by substituting the original pBR322 BglI-BamHI fragment with the similar BglI-BamHI fragment from pUC13 and subsequent insertion of the S.pombe TPI gene (POT) (U.S. patent application Ser. No. 614,734 filed on May 25, 1984) as a BamHI-SalI fragment to give CPOT. C1/1 is derived from pJDB 248, Beggs et al., Nature 275, 104–109 (1978) as described in EP patent application 0103409A.

EXAMPLE 5

Transformation

S. cerevisae strain MT118 (a, leu 2, ura 3, trp 1) was grown on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) to an OD_{600} of 2.1 100 ml of culture was harvested by

centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of (1.2M sorbitol, 25 mM Na_2EDTA pH=8.0, 6.7 mg/ml dithiotreitol). The suspension was incubated at 30° C. for 15 minutes, centrifuged and the cells resuspended in 10 ml of (1.2M sorbitol, 10 mM Na_2EDTA, 0.1M sodium citrate pH=5.8, 2 mg Novozym ® 234 enzyme). The suspension was incubated at 30° C. for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2M sorbitol and in 10 ml of CAS (1.2M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (Tris=Tris(hydroxymethyl)-aminometan) pH=7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 μg of plasmid pMT344 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris pH=7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 μg/ml leucine) and incubated at 30° C. for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2M sorbitol. 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) with leucine omitted and containing 1.2M sorbitol plus 2.5% agar) at 52° C. was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30° C., reisolated and used to start liquid cultures. One such transformant MT350 (=MT 118/pMT344) was chosen for further characterization.

Plasmid pMT475 was transformed into *S. cerevisiae* strain MT 362 (α, leu2) by the same procedure as above, and the transformant MT371 (=MT362/pMT475) isolated.

Transformation of pMT479 into strain E2-7BXE11-3C (a/α, Δtpi/Δtpi, pep 4-3/pep 4-3; this strain will be referred to as MT501) was performed as above with the following modifications: 1) prior to transformation strain MT501 was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an OD_{600} of 0.6. 2) the SOS solution contained YPGaL instead of YPD. One transformant MT519 (=MT501/pMT479) was chosen for further characterization.

The transformed microorganisms MT 350, MT 371 and MT 519 were deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), Griesebachstrasse 8, D-3400 Göttingen, on May 15, 1984 and accorded the reference numbers DSM 2957, DSM 2958, and DSM 2959, respectively.

EXAMPLE 6

Expression of B(1-29)-A(1-21) insulin in yeast

Strains MT350 (DSM 2957) and MT371 (DSM 2958) were grown in synthetic complete medium SC (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981) with leucine omitted. For each strain, two 1 liter cultures in 2 liter baffled flasks were shaken at 30° C. until they reached OD_{600nm} of 7 to 10. They were then centrifuged and the supernatant removed for further analysis.

Strain MT519 (DSM 2959) was grown similarly but on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) and to an OD_{600nm} of 15, centrifuged and the supernatant separated for analysis as above.

EXAMPLE 7

Expression of B(1-29)-A(1-21) insulin in yeast strain MT350 (DSM 2957)

Yeast strain MT350 (DSM 2957) was grown as previously described in example 6 and expression products from 1100 ml of supernatant from this strain were isolated as follows:

10 g of LiChroprep ® RP-18 (Merck, art. 9303) were washed 3 times with 50 mM NH_4HCO_3, 60% EtOH and thereafter packed in a 6×1 cm column. The column was equilibrated with 50 ml of 50 mM NH_4HCO_3. 55 ml of 96% EtOH were added to 1100 ml of the yeast supernatant, and the mixture was applied to the column overnight (flow: 70 ml/h).

The column was washed with 10 ml of 0.5M NaCl and 10 ml of H_2O, and the peptides were eluted with 50 mM of NH_4HCO_3, 60% EtOH. The eluate (5 ml) was concentrated by vacuum centrifugation to 1.4 ml (to remove the ethanol), and the volume was adjusted to 10 ml with 25 mM of HEPES buffer pH=7.4. The sample was applied to an antiinsulin immunoabsorption column (AIS column) (2.5×4.5 cm) which had been washed 4 times with 5 ml of NaFAM-buffer (Heding, L., Diabetologia 8, 260-66, 1972) and twice with 5 ml of 25 mM HEPES-buffer prior to the application. After the application, the column was allowed to stand for 30 min. at room temperature and was thereafter washed 10 times with 4 ml of 25 mM HEPES buffer. The peptides were eluted with 20% HAc. The pH value of the eluate was adjusted to 7.0 with NH_4OH, and the pool was concentrated to 500 μl by vacuum rotation.

The sample from the previous step was further purified on HPLC on a 10μ Waters μBondopak C-18 column (3.9×300 mm). The A and B buffers were 0.1% TFA in H_2O and 0.70% TFA in MeCN, respectively. The column was equilibrated with 25% B (flow: 1.5 ml/min.) and the peptides were eluted with a linear gradient of MeCN (1%/min.) and detected at 276 nm. The yield in each step of the purification was determined by radioimmunoassay as previously described, and Table 2 summarizes the purification. The overall yield was 68%.

TABLE 2

Purification of expression products from yeast strain MT350 supernatant

Purification step	Volume (ml)	Immunoreactive B(1-29)-A(1-21) insulin (nmol)
Supernatant	1100	110[a]
RP-18	10	116
Anti-insulin Sepharose	0.5	116
HPLC	2.5	75

[a]Dilution effect was observed in this sample

Only one peak containing immunoreactive B(1-29)-A(1-21) insulin material was detected from the HPLC column. Peptide material from this peak was isolated and subjected to amino acid sequence analysis. The sequence analysis was performed with a Gas Phase sequencer (Applied Biosystem Model 470A) as described by Hewick, R. M. et al. (J. Biol. Chem. 256, 7990–7997, 1981). From the sequencing results it could be concluded that the expression products consisted of 3 peptides:

(Glu—Ala)$_2$-B(1-29)-A(1-21) insulin	89%
Glu—Ala-B(1-29)-A(1-21) insulin	2%
B(1-29)-A(1-21) insulin	9%

The peptides were present in the relative amount as indicated.

EXAMPLE 8

Expression of B(1-29)-A(1-21) insulin in yeast strain MT371 (DSM 2958)

Yeast strain MT371 (DSM 2958) was grown as previously described in example 6 and expression products from 665 ml of supernatant from this strain were isolated as described in Example 7. The overall yield was 50 mmol, corresponding to 39%. Peptide material was isolated from the HPLC column and sequenced as described in Example 7. From the sequence results (18 residues from the N-terminal) it could be concluded that the peptide was homogeneous B(1-29)-A(1-21) insulin.

Comparison of these results to the results obtained in Example 7 indicates the advisability of removing the Glu-Ala-Glu-Ala sequence from the C-terminal of the MFα1 leader. It appears from Example 7 that the yeast dipeptidase enzyme does not function very efficiently in splitting off the Glu-Ala and Glu-Ala-Glu-Ala from the B(1-29)-A(1-21) insulin prior to secretion of the insulin precursor from the yeast cells.

EXAMPLE 9

Expression of B(1-29)-A(1-21) insulin in yeast strain MT519 (DSM 2959)

Yeast strain MT519 (DSM 2959) was grown as previously described in example 6 and expression products from 70 ml of supernatant were isolated as described in example 7. The overall yield was 116 mmol, corresponding to 57%. The peptide was sequenced as described in Example 7. As judged from the 42 residues identified from the N-terminal end, the peptide was homogeneous B(1-29)-A(1-21) insulin. Approximately 5 nmol of peptide was hydrolyzed in 100 µl 6N HCl for 24 h at 110° C. The hydrolysate was analyzed on a Beckman Model 121M amino acid analyser. The following amino acid composition was found:

TABLE 3

Amino acid analysis of purified B(1-29)-A(1-21) insulin

Amino acid	Found	Theoret.	Amino acid	Found	Theoret.
Asx*	2.97	3	Val	3.37	4
Thr	1.77	2	Ile	1.65	2
Ser	2.45	3	Leu*	5.65	6
Glx*	6.68	7	Tyr	3.51	4
Pro	1.33	1	Phe*	2.73	3
Gly*	3.95	4	Lys*	0.95	1
Ala*	1.22	1	His*	1.84	2
Cys 0.5	4.54	6	Arg*	1.13	1

*amino acid used for normalization.

EXAMPLE 10

Construction of a yeast plasmid pMT610 for expression of B(1-29)-Ala-Ala-Lys-A(1-21)

A 4.3 kb EcoRV-XbaI and a 3.3 kb EcoRI-EcoRV fragment from pMT342 (see example 3) were ligated to a 0.6 kb EcoRI-XbaI fragment of pM215 (see example 3). The obtained plasmid pMT462 harbours the insert MFα1 leader (minus Glu-Ala-Glu-Ala)-B-C-A. For converting the B-C-A encoding fragment into a B(1-29)-Ala-Ala-Lys-A(1-21) encoding fragment the modified site specific mutagenesis procedure (K. Norris et al., ibid.) was used. A 0.6 kb EcoRI-XbaI fragment from pMT462 encoding MFα1 leader (minus Glu-Ala-Glu-Ala)-B-C-A was inserted into M13 mp10 RF phage cut with XbaI-EcoRI. Single strand M13 phage containing the above EcoRI-XbaI insert was incubated with a 30mer d(TTCACAATGCCCTTAGCGGCCTTGGGTGTG) primer (KFN15) and the "universal" 15-mer M13 primer d(TCCCAGTCACGACGT) (see example 1), heated to 90° C. for 5 minutes and slowly cooled to room temperature in order to allow annealing. Then partly double stranded DNA was made by addition of a d-NTP-mix, Klenow Polymerase and T4 ligase. After phenol extraction, ethanol precipitation and resuspension, the DNA was cut with restriction enzymes ApaI, XbaI and EcoRI. After another phenol extraction, ethanol precipitation and resuspension, the DNA was ligated to EcoRI-XbaI cut pUC13. The ligation mixture was transformed into an *E. coli* (r$^-$m$^+$) strain and plasmids were prepared from a number of transformants. Plasmid preparations were cut with EcoRI and XbaI and those preparations showing bands at both 0.5 and 0.6 kb were retransformed into *E. coli*. From the retransformation a transformant harbouring only pUC13 with a 0.5 kb insert was selected. The sequence of the EcoRI-XbaI insert of this plasmid, pMT598, was then confirmed by the Maxam-Gilbert method to encode MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21). The XbaI-EcoRI insert from pMT598 was provided with TPI promotor and TPI terminator by ligation of a 0.5 kb XbaI-EcoRI fragment of pMT598 with a 5.5 kb XbaI-EcoRI fragment of pT5. The construction of pT5 harbouring the insert TPIp-MFα1 leader B-C-A-TPI$_T$ is illustrated in FIG. 8. The resulting plasmid pMT 601 containing the insert TPIp-MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21)-TPI$_T$ was cut with BamHI and partially with SphI and the 2.1 kb fragment was inserted in CPOT cut with BamHI and SphI. The resulting plasmid pMT610 was used for transformation of yeast.

EXAMPLE 11

Construction of a yeast plasmid pMT639 for expression of B(1-29)-Ser-Lys-A(1-21)

The BCA encoding fragment from pMT462 (see example 10) was converted into B(1-29)-Ser-Lys-A(1-21) by a procedure analogous with the procedure described in example 10 by site specific mutagenesis with a mixture of 27-mer d(TCCACAATGCCCTTAGACTTGGGTGTG) primer KFN36 and the "universal" 15-mer M13 primer. After filling in with Klenow polymerase and ligation with T4 ligase the partly double stranded DNA was digested with ApaI, EcoRI and XbaI and ligated with the 5.5 kb XbaI-EcoRI fragment from plasmid pT5 (see example 10). After transformation and retransformation into *E. coli*, a plasmid pMT 630 containing the insert MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21) was isolated and the sequence of the insert confirmed. The further procedure for obtaining plasmid pMT639 containing the insert TPIp-MFα1 (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPI$_T$ was as described in example 10. The construction of pMT639 is illustrated in FIG. 9.

EXAMPLE 12

Expression of B(1-29)-Ala-Ala-Lys-A(1-21) in yeast strain MT 620

S. cerevisiae strain MT501 (see example 5) was transformed with pMT 610 as described for pMT479 in example 5. Transformant colonies were picked after 3 days at 30° C., reisolated and used to start liquid cultures. One such transformant MT 620=(MT501/pMT610) was chosen for further characterization. MT620 was deposited by the applicant with Deutsche Sammlung von Mikroorganism (DSM), on Jan. 16, 1985 and accorded the reference number DSM 3196.

MT 620 was grown on YPD medium. A two liter culture in 2 liter baffled flask was shaken at 30° C. to an OD_{600nm} of 15. After centrifugation the supernatant was removed for further analysis. The expression level determined by radioimmunoassay was 1.2 μmol/l. Expression products from 840 ml of supernatant were purified as described in Example 7. (RP-18 column, Anti-insulin Sepharose and HPLC). The overall yield was 100 nmol corresponding to about 10%. Peptide material was isolated from the HPLC-column and sequenced as described in Example 7. 35 Edman degradation cycles were carried out (Table 4). From the sequence results the position of the 3 amino acid residue chains (Ala-Ala-Lys) separating the B(1-29) and the A(1-21) chains was confirmed (see table 4).

TABLE 4

Sequence analysis of B(1-29)-Ala—Ala—Lys-A(1-21) isolated from the culture medium of strain MT 620.

Cyclus No.	PTH-amino acid residue	Yield (pmol)
1	Phe	3381
2	Val	1738
3	Asn	5169
4	Gln	2750
5	His	2045
6	Leu	1405
7	Cys	—
8	Gly	1372
9	Ser	345
10	His	1105
11	Leu	2228
12	Val	1963
13	Glu	1219
14	Ala	1514
15	Leu	1793
16	Tyr	1707
17	Leu	1354
18	Val	1765
19	Cys	—
20	Gly	882
21	Glu	1019
22	Arg	1100
23	Gly	1123
24	Phe	1492
25	Phe	2042
26	Tyr	1014
27	Thr	195
28	Pro	710
29	B_{29}Lys	1173
30	Ala	1026
31	Ala	885
32	Lys	1175
33	A_1Gly	552
34	Ile	518
35	Val	548

The average repetitive yield was 95.6%.

EXAMPLE 13

Expression of B(1-29)-Ser-Lys-A(1-21) in yeast strain MT643

S. cerevisiae strain MT501 was transformed with pMT639 as described for pMT479 in example 5.

One transformant MT643=(MT501/pMT639) was chosen for further characterization. MT643 was deposited by the applicant at DSM on Jan. 16, 1985 and accorded the reference No. DSM 3197.

MT643 was grown as described in example 12. After centrifugation the supernatant was removed for further analysis.

The expression level of the insulin precursor determined by radioimmunoassay was 1.6 μmol/l. Expression products from the supernatant from strain MT 643 was isolated as described in Example 7. The peptide material isolated from the HPLC column was submitted to sequence analysis as described in Example 7. From the sequence results (not shown) the position of the two amino acid residues chains (Ser-Lys) separating the B(1-29) and A(1-21) chains was confirmed.

EXAMPLE 14

Conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10M acetic acid. 0.26 ml of 1.54M Thr(Bu^t)-OBu^t in N,N-dimethylacetamide was added. The mixture was cooled to 12° C. 2.8 mg of trypsin dissolved in 0.035 ml of 0.05M calcium acetate was added. After 72 hours at 12° C., the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin was 64% by HPLC.

EXAMPLE 15

Conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10M acetic acid. 0.26 ml of 1.54M Thr-OMe in a mixture of dimethyl sulphoxide and butane-1,4 diol 1/1 (v/v) was added. 1 mg of lysyl endopeptidase from Achromobacter lyticus (Wako Pure Chemical Industries, Osaka, Japan) in 0.07 ml of water was added. After 120 hours at 25° C., the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation, and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin was 75% by HPLC.

EXAMPLE 16

Conversion of B(1-29)-Ser Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg B(1-29)-Ser-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N-dimethylformamide (v/v) in water. 0.2 ml of 2M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12° C. 2 mg of trypsin in 0.05 ml 0.05M calcium acetate was added. After 24 hours at 12° C., the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ser-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 85% by HPLC.

EXAMPLE 17

Conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg B(1-29)-Ala-Ala-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N-dimethylformamide (v/v) in water. 0.2 ml of 2M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12° C. 2 mg of trypsin in 0.05 ml 0.05M calcium acetate was added. After 96 hours at 12° C., the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 84% by HPLC.

EXAMPLE 18

Preparation of human insulin from various human insulin esters

The human insulin esters in the crude acetone precipitates were purified by gelfiltration and anion exchange chromatography as described in Methods in Diabetes Research vol. 1, p. 407–408 (Eds. J. Larner and S. Pohl (John Wiley Sons, New York, 1984)). The method was applicable to any of the 3 human insulin esters. The cleavages of the various ester groups, rendering human insulin in nearly 100% yields, were carried out by hydrolysis of Thr-OMe(B30) human insulin and by acidolysis with trifluoroacetic acid of Thr(Bu^t)-OBu^t(B30) human insulin and of Thr-OBu^t(B30) human insulin as described ibid. p. 409.

We claim:

1. A DNA-sequence comprising a sequence encoding an insulin precursor of the formula:

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n=0 to 33, m=0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain —X_n-Y- does not contain two adjacent basic amino acid residues.

2. A DNA-sequence according to claim 1 wherein m is 0.

3. A DNA-sequence according to claim 1 wherein m is 1 and n is 1–8.

4. A DNA-sequence according to claim 1 wherein said insulin precursor is

B(1-29)-Ala-Ala-Lys-A(1-21).

5. A DNA-sequence according to claim 1 wherein said insulin precursor is B(1-29)-Ser-Lys-A(1-21).

6. A replicable expression vehicle capable of expressing in yeast a DNA-sequence encoding an insulin precursor of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n=0 to 33, m=0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain —X_n-Y- does not contain two adjacent basic amino acids.

7. A replicable expression vehicle according to claim 6 comprising the plasmid pMT344.

8. A replicable expression vehicle according to claim 6 comprising the plasmid pMT475.

9. A replicable expression vehicle according to claim 6 comprising the plasmid pMT479.

10. A replicable expression vehicle according to claim 6 comprising the plasmid pMT610.

11. A replicable expression vehicle according to claim 6 comprising the plasmid pMT639.

12. A yeast strain transformed with a replicable expression vehicle capable of expression in yeast a DNA-sequence encoding an insulin precursor of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n=0 to 33, m=0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain —X_n-Y- does not contain two adjacent basic amino acids.

13. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT344.

14. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT475.

15. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT479.

16. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT610.

17. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT639.

18. A process for producing an insulin precursor of the formula:

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n expressible amino acid residues, Y is Lys or Arg, n=0 to 33, m=0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain —X_n-Y- does not contain two adjacent basic amino acid residues; said process comprising the steps of:

culturing a transformant yeast strain in a suitable nutrient medium, said transformant yeast strain including a replicable expression vehicle capable of expressng a DNA-sequence encoding said insulin precursor, and recovering said insulin precursor.

19. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 2957.

20. A process for producing an insulin precursor according to claim 19, wherein said insulin precursor has the formula B(1-29)-A(1-21).

21. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 2958.

22. A process for producing an insulin precursor according to claim 21, wherein said insulin precursor according to claim 21, wherein said insulin precursor has the formula

B(1-29)-A(1-21).

23. A process for producing an insulin precursor according to claim 18, wherein yeast strain is DSM 2959.

24. A process for producing an insulin precursor according to claim 23, wherein said insulin precursor has the formula B(1-29)-A(1-21).

25. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 3196.

26. A process for producing an insulin precursor according to claim 25, wherein said insulin precursor has the formula B(1-29)-Ala-Ala-Lys-A(1-21).

27. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 3197.

28. A process for producing an insulin precursor according to claim 27, wherein said insulin precursor has the formula B(1-29)-Ser-Lys-A(1-21).

29. A process for preparing human insulin comprising the steps of:

transforming a yeast strain with a replicable expression vehicle capable of expressing a DNA-sequence encoding an insulin precursor of the formula

B(1-29)-$(X_n$-Y)$_m$-A(1-21),

wherein X_n is a peptide chain with n expressible amino acid residues, Y is Lys or Arg, n=0 to 33, m=0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the chain —X_n-Y- does not contain two adjacent basic amino acids;

culturing said transformed yeast strain in a suitable nutrient medium so that expression of said insulin precursor occurs;

recovering said expressed insulin precursor from said nutrient medium; and

converting said insulin precursor into human insulin.

30. Human insulin precursors of the general formula

B(1-29)-X_n-Y-A(1-21),

wherein X_n is a peptide chain with n expressible amino acid residues, n=0 to 33, Y is Lys or Arg, B(1-21) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain —X_n-Y- does not contain two adjacent, basic amino acid residues.

31. A human insulin precursor according to claim 30 having the formula

B(1-29)-Ala-Ala-Lys-A(1-21).

32. A human insulin precursor according to claim 30 having the formula

B(1-29)-Ser-Lys-A(1-21).

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EGULAR UTILITY

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FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
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[illegible]-SEQUENCES OF [illegible] SYNTHETIC INSULIN PRECURSORS AND PROCESS FOR
REPARING THE [illegible] PRECURSORS TO HUMAN INSULIN

U.S. DEPT. of COMM.-Pat. & TM Office — PTO-436L (rev. 10-78)

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435	253 172.3 68 70 320 28 27 303 808 2 17 18 13 29 69	3/3/88 12/29/88	JC JC
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1-18

INDEX OF CLAIMS

Claim Final	Claim Original	Date	Claim Final	Claim Original	Date
	1	✓		26	✓
	2	✓		27	✓
	3	✓		28	✓
	4	✓		29	✓
	5	✓		30	✓
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	7	✓		32	✓
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	18	✓		43	
	19	✓		44	
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	23	✓		48	
	24	✓		49	
	25	✓		50	

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Transmitted herewith for filing is the patent application of

Inventor: JAN MARKUSSEN et al

For: DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN etc.

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TITLE: DNA-sequence Encoding Biosynthetic Insulin Precursors and Process for Preparing the Insulin Precursors and Human Insulin.

ABSTRACT: Human insulin precursors containing the peptide chain B(1-29)-A(1-21) of human insulin and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)-chain are prepared by culturing a yeast host transformed with a replicable expression vehicle capable of expressing a DNA-sequence encoding the insulin precursor. The bridging chain is preferably relatively short and contains preferably from 2 to 8 amino acid residues. The bridging chain must not contain two adjacent basic amino acid residues (Lys or Arg) and has one Lys or Arg connected to the amino terminus of the A(1-21)-chain. Human insulin is prepared from the insulin precursors by in vitro conversion.

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This invention relates to biosynthetic insulin. More specifically, the invention is directed to DNA-sequences encoding biosynthetic insulin precursors and to the preparation of such insulin precursors which are convertible into biosynthetic human insulin by in vitro conversion.

BACKGROUND OF THE INVENTION

In the past insulin has been synthezised (from synthetic A- and B-chains) or re-synthesized (from naturally derived A- and B-chains) by combining the two chains in an oxidation process whereby the 6 cysteine sulfhydryl groups of the reduced chains (4 in the A-chain, 2 in the B-chain) are converted into disulfide bonds. By this method disulfide bonds are formed largely at random, meaning that the yield of insulin with disulfide bridges correctly positioned between cysteine residues A-6 and A-11, A-7 and B-7, and A-20 and B-19, respectively, is very low.

Following the discovery of proinsulin as a biological precursor of insulin it was observed that the A- and B-polypeptide moieties of the linear-chain totally reduced proinsulin (those moieties corresponding to the A- and B-chains of insulin, respectively) could be oxidatively combined with much less randomization of the disulfide bonds to give a substantially higher yield of correctly folded proinsulin as compared with the combination of free A- and B-chains (D.F. Steiner et al.: Proc.Nat.Acad.Sci. 60 (1968), 622). Albeit high yields were obtained only at proinsulin concentrations too low to make the process feasible on a preparative scale, the function of the C- (i.e. connecting peptide) moiety of the B-C-A polypeptide sequence of proinsulin, namely that of bringing the 6 cysteine residues into spatial positions favorable for correct oxidation into proinsulin, was clearly demonstrated.

The proinsulin formed may function as an *in vitro* precursor of insulin in that the connecting peptide is removable by enzymatic means (W. Kemmler *et al.*: J.Biol.Chem. *246* (1971), 6786).

Subsequently it has been shown that proinsulin-like compounds having shorter linking moieties than the C-peptide and flanked at both ends by specific enzymatic or chemical cleavage sites (the so-called miniproinsulins (A. Wollmer et al., Hoppe-Seyler's Z. Physiol.Chem. 355 (1974), 1471 - 1476 and Dietrich Brandenburg *et al.*, Hoppe-Seyler's Z. Physiol.Chem. 354 (1973), 1521 - 1524)) may also serve as insulin precursors.

Endeavours to provide biosynthetic insulins, particularly that identical to the human species, have followed the same strategic pathways as those to synthetic insulin. The insulin A- and B-chains have been expressed in separate host organisms, isolated therefrom and then combined as described *supra* (R.E. Chance *et al.*: Diabetes Care *4* (1982), 147). Microorganisms have been transformed with cloning vectors encoding preproinsulin or proinsulin which may be secreted as such (W. Gilbert *et al.*: European Patent Publ. No. 6694) or accumulated intracellularly as hybrid gene products (D.V. Goeddel *et al.*: European Patent Publ. No. 55945). The miniproinsulin pathway has also been attempted (D.V. Goeddel, *supra*).

Procuring the A- and B-chains in separate fermentation processes followed by combination of the chains is inherently impractical. The dual fermentation inconvenience may be overcome by choosing the proinsulin or miniproinsulin strategy. However, the use of a proinsulin as the biosynthetic insulin precursor may entail certain disadvantages. The proinsulin, whether excreted into the fermentation liquid as such or accumulated intracellularly in the host organism, possibly as a hybrid gene product, is likely to contain substantially randomized disulfide bonds. The refolding of such "scrambled" products into correctly folded proinsulin may be conducted either directly (H.-G. Gattner *et al.*: Danish Patent Application No. 4523/83) or via the single chain hexa-S-sulfonate (F.B. Hill: European Patent Publ. No. 37255). The refolding

process usually entails some degree of polymerization and hence the inconvenience of using laborious purification steps during recovery.

In addition, insulin precursors of the proinsulin type are prone to undergo enzymatic degradation, either within the host cells or following its excretion into the fermentation broth. In yeast it has been shown that human proinsulin is particularly sensitive to enzymatic cleavages at the two dibasic sequences (Arg31-Arg32 and Lys64-Arg65). Apparently these cleavages occur before the establishment of the S-S bridges, resulting in the formation of C-peptide, A-chain and B-chain.

OBJECT OF THE INVENTION AND SUMMARY THEREOF

The object of the present invention is to circumvent these disadvantages by devising biosynthetic insulin precursors which are generated largely with correctly positioned disulfide bridges between the A- and B-moieties and, furthermore, substantially more resistant to proteolytic degradation than the biosynthetic insulin precursors known heretofore.

A single chain insulin precursor consisting of a shortened insulin B-chain from Phe^{B1} to Lys^{B29} continuing into a complete A-chain from Gly^{A1} to Asn^{A21}, B(1-29)-A(1-21), is known (Jan Markussen, "Proteolytic degradation of proinsulin and of the intermediate forms",: Proceedings of the Symposium on Proinsulin, Insulin and C-Peptide, Tokushima, 12 - 14 July, 1978, Editors: S. Baba et al.). This insulin precursor B(1-29)-A(1-21) is prepared by a semisynthetic process from porcine insulin. First the insulin B(1-29) and A(1-21) chains were prepared and coupled to form a linear peptide B(1-29)-A(1-21). This compound in the hexathiol form was oxidized in vitro rendering the single chain des-(B30) insulin molecule.

The present invention is based on the surprising discovery that the above single chain insulin precursor B(1-29)-A(1-21) and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)-chain are expressed in high yields and

with correctly positioned disulfide bridges when culturing yeast strains transformed with DNA-sequences encoding such insulin precursors are cultured.

According to a first aspect of the present invention there is provided a DNA-sequence encoding insulin precursors of the formula

$$B(1-29)-(X_n-Y)_m-A(1-21) \qquad I$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n is an integer from 0 to 33, m is 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) is the A chain of human insulin, with the proviso that the peptide chain $-X_n-Y-$ does not contain two adjacent basic amino acid residues (i.e. Lys and Arg).

Preferred insulin precursors of the above formula I are B(1-29)-A(1-21), i.e. m = 0 in formula I, and compounds with a relative short bridging chain between the B(1-29)- and the A(1-21)- chain.

When m = 1, then n is preferably 1-33, more preferably 1-15, 1-8 or 1-5 and most preferably 1-3 or 1-2. X may preferably be selected from the group consisting of Ala, Ser and Thr, the individual X's being equal or different. Examples of such preferred compounds are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a second aspect of the present invention there is provided a replicable expression vehicle capable of expression of a DNA-sequence comprising a sequence encoding the insulin precursors of formula I in yeast.

The expression vehicle may be a plasmid capable of replication in the host microorganism or capable of integration into the host organism chromosome. The vehicle employed may code for expression of repeated sequences of the desired DNA-sequence, each separated by selective cleavage sites.

According to a third aspect of the present invention there is provided a process for producing insulin precursors of formula I in yeast wherein a transformant yeast strain including at least one expression vehicle capable of expressing the insulin precursors is cultured in a suitable nutrient medium followed by isolation of the insulin precursors.

According to a fourth aspect of the present invention there are provided novel human insulin precursors. Such novel human insulin precursors have the following general formula

$$B(1-29)-X_n-Y-A(1-21) \qquad II$$

in which the different symbols have the above mentioned definitions. Preferred novel insulin precursors are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a fifth aspect of the present invention there is provided a yeast strain transformed with an expression vehicle capable of expressing a DNA-sequence comprising a sequence encoding the above insulin precursors in yeast.

The insulin precursors may be expressed with additional protein proceeding the insulin precursor. The additional protein may have the function of protecting the insulin precursor against, e.g. in vivo degradation by endogeneous enzymes or of providing information necessary to transport the desired protein into the periplasmic space and finally across the cell wall into the medium.

The additional protein contains a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors enabling subsequent splitting off of the additional protein either by the microorganism itself or by later enzymatical or chemical cleavage.

Accordingly the present invention includes a DNA-sequence encoding the above insulin precursors and further comprising an additional DNA-sequence positioned upstream to the sequence encoding the insulin precursors and encoding an additional amino acid-sequence containing a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors.

According to a preferred embodiment of the present invention the additional amino acid sequence comprises at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor.

When the insulin precursor is expressed in yeast the additional amino acid-sequence may contain two basic amino acids (e.g. Lys-Lys, Arg-Arg, Lys-Arg or Arg-Lys) adjacent to N-terminal of the B(1-29)-chain of the insulin precursor, yeast being able to cleave the peptide bond between the basic amino acids and the precursor. Also a Glu-Ala or Asp-Ala cleavage site adjacent to the desired protein enables separation of the additional amino acid sequence by the yeast itself by means of a dipeptidase enzyme produced by the yeast.

The insulin precursors may be secreted with an amino acid-sequence linked to the B(1-29)-chain of the precursors provided that this amino acid sequence contains a selective cleavage site adjacent to the B(1-29)-chain for later splitting of the superfluous amino acid sequence. If the insulin precursors do not contain methionine cyanogen bromide cleavage at methionine adjacent to the desired protein would be operative. Likewise, arginine- and lysine-cleavage sites adjacent to the desired protein enables cleavage with trypsinlike proteases.

For secretion purposes the DNA-sequence encoding the insulin precursors may be fused to an additional DNA-sequence coding for a signal peptide. The signal peptide is cleaved off by the transformant microorganism during the secretion of the expressed protein product from the cells ensuring a more simple isolation of the desired product. The secreted product may be the insulin precursor or may contain an additional N-terminal amino acid-sequence to be removed later as explained above.

Secretion may be provided by including in the expression vehicle the yeast MFα1 leader sequence (Kurjan, J. and Herskowitz, I., Cell 30, (1982), 933 - 943) and according to a further preferred embodiment of the present invention the additional amino acid-sequence positioned upstream to the sequence encoding the insulin precursors comprises the yeast MFα1 leader coding sequence or part thereof.

The expression of the desired DNA-sequence will be under control of a promoter sequence correctly positioned to the DNA-sequence encoding the desired protein product to result in expression of the desired protein in the host organism. Preferably a promoter from a gene indigenous to the host organism may be

used. The DNA-sequence for the desired protein will be followed by a transcription terminator sequence, preferably a terminator sequence from a gene indigenous to the host organism. If yeast is used as host organism the promoter and terminator sequences are preferably the promoter and terminator of the triose phosphase isomerase (TPI) gene, respectively.

Other promoters may be utilized such as the phosphoglycerate kinase (PGK1)- and the MFα1-promoter.

The present invention further comprises a method for preparing human insulin by which a yeast strain is transformed with a replicable expression vehicle comprising a DNA-sequence encoding the insulin precursors of the above formula I, the transformed yeast strain is cultured in a suitable nutrient medium, the insulin precursors are recovered from the culture medium and converted in vitro into human insulin.

The insulin precursors according to the present invention may be converted into mature human insulin by transpeptidation with an L-threonine ester in the presence of trypsin or a trypsin derivative as described in the specification of U.S. Patent 4,343,898 (~~Danish patent application 574/80~~) (the disclosure of which is incorporated by reference hereinto) followed by transformation of the threonine ester of human insulin into human insulin by known processes.

If the insulin precursors are secreted with an additional amino acid sequence adjacent to the N-terminal of the B(1-29)-chain such amino acid sequence should either be removed in vitro before the transpeptidation or should contain at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain as trypsin will cleave the peptide bond between the basic amino acid and the amino group of Phe^{B1} during the transpeptidation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate a preferred embodiment of the present invention.

Fig. 1 illustrates the preparation of plasmid pMT344,
fig. 2 illustrates the preparation of plasmid pMT475,
fig. 3 illustrates the preparation of plasmid pMT212,
fig. 4 illustrates the preparation of plasmid pMT479
fig. 5 illustrates the preparation of plasmid pMT319,
fig. 6 illustrates the preparation of plasmid pMT598,
fig. 7 illustrates the preparation of plasmid pMT610,
fig. 8 illustrates the preparation of plasmid pT5, and
fig. 9 illustrates the preparation of plasmid pMT639.

In the drawings and part of the following description the expression B' is used instead of B(1-29) and A instead of A(1-21). Accordingly the expression B'A is equivalent to the expression B(1-29)-A(1-21).

DETAILED DESCRIPTION

1. Preparation of a gene coding for human proinsulin B-C-A

Total RNA purified (Chirgwin, J.M. Przybyla, A.E., McDonald, R.J. & Rutter, W.J., Biochemistry 18, (1979) 5294 - 5299) from human pancreas was reverse transcribed (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J.F. & Marcker, K.A., Proc.Natl.Acad.Sci. USA 80, (1983), 2866 - 2869) with AMV reverse transcriptase and d(GCTTTATTCCATCTCTC) as 1. strand primer. After preparative urea-polyacrylamide gel purification of the human proinsulin cDNA, the second strand was synthesized on this template with DNA polymerase large fragment and d(CAGATCACTGTCC) as 2nd strand primer. After S1 nuclease digestion the human proinsulin ds. cDNA was purified by polyacrylamide gel electrophoresis, tailed with terminal transferase and cloned in the PstI site on pBR327 (Sorberon et al., Gene 9, (1980), 287 - 305) in E. coli. A correct clone harbouring a plasmid containing a gene encoding human proinsulin B-C-A was identified from the recombinants by restriction endonuclease analysis and confirmed by nucleotide sequencing

(Maxam, A., & Gilbert, W., Methods in Enzymology, 65 (1980), 499 - 560. Sanger, F., Nicklen, S. & Coulson, A.R., Proc.Natl.Acad.Sci. USA 74, (1977), 5463 - 5467).

2. Preparation of genes coding for precursors of human insulin.

The gene encoding B(1-29)-A(1-21) of human insulin was made by site specific mutagenesis of the human proinsulin sequence with a 75bp in frame deletion in the C-peptide coding region inserted into a circular single stranded M-13 bacteriophage vector. A modified procedure (K. Norris et al., Nucl.Acids.Res. 11 (1983) 5103 - 5112) was used in which a chemically synthesized 19-mer deletion primer was annealed to the M13 template. After a short enzymatic extension reaction a "universal" 15-mer M13 dideoxy sequencing primer was added followed by enzymatic extension and ligation. A double stranded restriction fragment (BamHl-Hind III) was cut out of the partly double stranded circular DNA and ligated into pBR322 cut with BamHI and Hind III.

The obtained ligation mixture was used to transform E. coli and transformants harbouring a plasmid pMT319 containing the gene encoding B(1-29)-A(1-21) of human insulin were identified.

Genes encoding B(1-29)-Ala-Ala-Lys-A(1-21) and B(1-29)-Ser-Lys-A(1-21) were made accordingly by insertion of a fragment encoding MFα1-B-C-A in the M-13 bacteriophage and site specific mutagenesis of the human proinsulin sequence with chemically synthesized 30-mer and 27-mer deletion primers, respectively, and the above mentioned "universal" 15-mer M13 dideoxy sequencing primer. A double stranded restriction fragment (Xbal-EcoRl) was cut out of the partly double stranded circular DNA and ligated into pUC13 and pT5, respectively. By transformation and retransformation of E. coli, transformants harbouring a plasmid pMT598 containing the gene encoding B(1-29)-Ala-Ala-Lys-A(1-21) and pMT630 containing the gene encoding B(1-29)-Ser-Lys-A(1-21) were identified.

A gene encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was made in a similar way as described above by insertion of a fragment encoding MFα1-B(1-29)-A(1-21) in a M13

mpll bacteriophage and site specific mutagenesis of the B(1-29)-A(1-21) sequence with a chemically synthesized 46-mer deletion primer (5'-CACACCCAAGACTAAAGAAGCTGAAGACTTGCAAAGAGGCATTGTG-3') and the "universal" primer. Also, by a similar procedure a gene encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-A(1-21) was constructed.

3. Plasmid constructions.

The gene encoding B(1-29)-A(1-21) of human insulin (B'A) was isolated as a restriction fragment from pMT319 and combined with fragments coding for the TPI promoter (TPI_p) (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434), the MFα1 leader sequence (J. Kurjan and I. Herskowitz,. Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) and the transcription termination sequence from TPI of S.cerevisiae (TPI_T). These fragments provide sequences to ensure a high rate of transcription for the B'A encoding gene and also provide a presequence which can effect the localization of B'A into the secretory pathway and its eventual excretion into the growth medium. This expression unit for B'A (TPI_p-MFα1 leader - B'A - TPI_T was then placed on a plasmid vector containing the yeast 2μ origin of replication and a selectable marker, LEU 2, to give pMT344, a yeast expression vector for B'A.

During in vivo maturation of α-factor in yeast, the last (C-terminal) six amino acids of the MFα1 leader peptide (Lys-Arg-Glu-Ala-Glu-Ala) are removed from the α-factor precursor by the sequential action of an endopeptidase recognizing the Lys-Arg sequence and an aminodipeptidase which removes the Glu-Ala residues (Julius, D. et al. Cell 32 (1983) 839 - 852). To eliminate the need for the yeast aminodipeptidase, the sequence coding for the C-terminal Glu-Ala-Glu-Ala of the MFα1 leader was

removed via in vitro mutagenesis. The resulting yeast expression plasmid, pMT475, contains the insert coding for TPI_p-MFα1 leader (minus Glu-Ala-Glu-Ala) - B'A - TPI_T.

In a preferred construction the modified expression unit was transferred to a stable, high copy number yeast plasmid CPOT, (ATCC No. 39685), which can be selected merely by the presence of glucose in the growth medium. The resulting yeast expression vector for B'A was numbered pMT479.

The fragment encoding MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21) was isolated as a restriction fragment from pMT598 and combined with fragments coding for the TPI promoter and the TPI terminator and transferred to the above mentioned high copy number yeast plasmid CPOT. The resulting yeast expression vector for B(1-29)-Ala-Ala-Lys-A(1-21) was numbered pMT610.

The fragment containing the insert TPI_p- MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPI_T was isolated as a restriction fragment from pMT630 and transferred into CPOT. The resulting yeast expression vector for B(1-29)-Ser-Lys-A(1-21) was numbered pMT639.

The fragment containing the insert TPI_p- MFα1 leader-(minus Glu-Ala-Glu-Ala)-B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21)-TPI_T was inserted into a high copy number yeast plasmid DPOT, being a CPOT derivative containing a SphI-BamHI-fragment of pBR322 inserted into a SpHI-BamHI fragment of CPOT. The resulting yeast expression vector for B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was numbered pl126.

4. Transformation

Plasmids pMT344 and pMT475 were transformed into S. cerevisiae leu 2 mutants by selection for leucin prototrophy as described by Hinnen et al.(A. Hinnen, J.B. Hicks and G.R. Fink. Transformation of Yeast. Proc.Nat.Aca.Sci. 75 (1978) 1929).

Plasmids pMT479, pMT610, pMT639 and pl126 were transformed into S. cerevisiae strains carrying deletions in the TPI gene by selecting for growth on glucose. Such strains are normally unable to grow on glucose as the sole carbon source and

grow very slowly on galactose lactate medium. This defect is due to a mutation in the triose phosphate isomerase gene, obtained by deletion and replacement of a major part of this gene with the S. cerevisiae LEU 2 gene. Because of the growth deficiencies there is a strong selection for a plasmid which contains a gene coding for TPI. pMT479 contains the Schizo. pombe TPI gene.

5. Expression of human insulin precursors in yeast

Expression products of human insulin type were measured by radioimmunoassay for insulin as described by Heding, L. (Diabetologia 8, 260 - 66, 1972) with the only exception that the insulin precursor standard in question was used instead of an insulin standard. The purity of the standards were about 98% as determined by HPLC and the actual concentration of peptide in the standard was determined by amino acid analysis. The expression levels of immunoreactive human insulin precursors in the transformed yeast strains are summarized in Table 1.

Table 1

Expression levels of immunoreactive human insulin precursors in yeast.

Yeast strain	Plasmid	Construct	Immunoreactive insulin precursor (nmol/l supernatant)
MT 350 (DSM 2957)	pMT 344	B(1-29)-A(1-21)	100
MT 371 (DSM 2958)	pMT 475	B(1-29)-A(1-21)	192
MT 519 (DSM 2959)	pMT 479	B(1-29)-A(1-21)	2900
MT 620 (DSM 3196)	pMT 610	B(1-29)-Ala-Ala-Lys-A(1-21)	1200 - 1600
MT 649 (DSM 3197)	pMT 639	B(1-29)-Ser-Lys-A(1-21)	1600
ZA 426	p1126	B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21)	200

The isolation and characterization of expression products are given in Examples 7 - 9 and 12 - 13.

6. Conversion of human insulin precursor into B30 esters of human insulin

The conversion of the human insulin precursors into human insulin esters can be followed quantitatively by HPLC (high pressure liquid chromatography) on reverse phase. A 4 x 300 mm "μBondapak C18 column" (Waters Ass.) was used and the elution was performed with a buffer comprising 0.2 M ammonium sulphate (adjusted to a pH value of 3.5 with sulphuric acid) and containing 26 - 50% acetonitrile. The optimal acetonitrile concentration depends on which ester one desires to separate from the insulin precursor. In case of human insulin methyl ester separation is achieved in about 26% (v/v) of acetonitrile.

Before the application on the HPLC column the proteins in the reaction mixture were precipitated by addition of 10 volumes of acetone. The precipitate was isolated by centrifugation, dried in vacuo, and dissolved in 1 M acetic acid.

EXPERIMENTAL PART

Example 1

Construction of a gene coding for B(1-29)-A(1-21)insulin

Materials and Methods

"Universal" 15-mer M13 dideoxy sequencing primer d(TCCCAGTCACGACGT), T4 DNA ligase and restriction enzymes were obtained from New England Biolabs. DNA polymerase I "Klenow fragment" and T_4 polynucleotide kinase were purchased from P-L Biochemicals. (γ-^{32}P)-ATP (7500 Ci/mmol) was obtained from New England Nuclear. The support for oligonucleotide synthesis was 5'-O-dimethoxytrityl N^2-isobutyryldeoxyguanosine bound via a 3'-O-succinyl group to aminomethylated 1% crosslinked polystyrene beads from Bachem.

Construction of M13 mp10 insHX PstΔphage:

The M13 mp10 derived phage mp10 insHX was constructed by cloning of the 284 bp large proinsulin coding Hind III-XbaI fragment, isolated from p285, into Hind III-XbaI cut M13 mp10 RF.M13 mp10 RF is available from P-L Biochemicals, Inc. Milwaukee, Wis. (Catalogue No. 1541).

M13 mp10 insHXΔPst was constructed from mp10 insHX,RF by complete PstI digestion followed by ligation and transformation of E. coli JM103. The resulting phage harbours the human proinsulin coding sequences, with a 75 bp in frame deletion in the C-peptide coding region. Single stranded phage was prepared as described (Messing, J. and Vieira, J. (1982) Gene 19, 269 - 276).

Oligodeoxyribonucleotide synthesis

The 19-mer deletion primer d(CACACCCAAGGGCATTGTG) was synthesized by the triester method on a 1% crosslinked polystyrene support (Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) Nucl.Acids Res. 10, 1755 - 1769). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an HPLC pump and a control module. The oligonucleotide was purified after deprotection by HPLC on a LiChrosorb RP18 column (Chrompack (Fritz, H.-J., Belagaje, R., Brown, E.L., Fritz, R.H., Jones, R.A., Lees, R.G., and Khorana, H.G. (1978) Biochemistry 17, 1257 - 1267).

5'-^{32}P-labelling of oligodeoxyribonucleotide

The 19-mer was labelled at the 5'end in a 60µl reaction mixture containing 50 mM Tris-HCl at pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 0.4% glycerol, 120 pmole ATP, 50 µCi of (γ-^{32}P)-ATP (10 pmole), 120 pmole of oligonucleotide and 30 units of T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min., and terminated by heating at 100°C for 3 min. The labelled oligonucleotide was separated from unreacted (γ-^{32}P)-ATP by chromatography on a column (1 x 8 cm) of Sephadex G50 superfine in 0.05 M triethylammonium bicarbonate at pH 7.5.

For colony hybridization the oligonucleotide was labelled without the addition of "cold" ATP as described (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J., and Marcker, K. (1983) Proc.Natl.Acad.Sci. USA 80, 2866 - 2869).

Oligodeoxyribonucleotide primed DNA synthesis

Single stranded M13 mp10 insHXΔPst (0.4 pmole was incubated with the 19-mer 5'-(^{32}P)-labelled oligodeoxyribonucleotide primer (10 pmole) in 20 µl of 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DDT for 5 min. at 55°C and annealed for 30 min. at 11°C. Then 9 µl of d-NTP-mix consisting of 2.2 mM of each dATP, dCTP, dGTP, dTTP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DDT was added followed by 7 units of E. coli DNA polymerase I (Klenow). The mixture was kept for 30 min. at 11°C and heated for 10 min. at 65°C. 15-mer universal primer for dideoxy sequencing (4 pmole) was added and the mixture heated at 65°C for an additional minute. After cooling to 11°C 26 µl of solution containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.8 mM of each dATP, dCTP, dGTP, dTTP, 2.4 mM ATP and 10^3 units of T4 ligase was added followed by 9.5 units of E. coli DNA polymerase I (Klenow). The final volume of the mixture was 64 µl. After incubation for 3 hours at 11°C 20 µl 4M sodium acetate was added, and the volume adjusted to 200 µl with TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The mixture was extracted twice with phenol/chloroform. 0.9 µg (0.3 pmole) of the purified large fragment of pBR322 cleaved with BamHI and Hind III was added as carrier DNA. After ether extraction of the aqueous phase, the DNA was isolated by ethanol precipitation.

Endonuclease digestion

The DNA, prepared as described above, was digested respectively with 16 and 20 units of restriction endonucleases BamHI and Hind III in a total volume of 22µl of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DDT, 4 mM spermidine). The mixture was extracted with phenol/chloroform

followed by ether and the DNA was isolated by ethanol precipitation and then dissolved in 12 µl H_2O. 2µl was used for electrophoresis on a 7M urea 6% polyacrylamide gel.

Ligation

To a part of the DNA (5 µl) was added a new portion of the purified large fragment of pBR322 cut with BamHI and Hind III (0.38 µg) and 400 units of T4 DNA ligase, in a total volume of 41 µl containing 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DDT, 40 µg/ml gelatine. Ligation was performed at 16°C for 16 hours.

Transformation

20.5 µl of the ligation mixture was used to transform $CaCl_2$ treated E. coli MC 1000 (r^-, m^+). The bacteria were plated on LB-agar plates and selected for resistance to ampicillin (100 µg/ml). 2.6 x 10^3 colonies per pmole of M13 mp10 insHXΔPst were obtained.

Colony hybridization

123 transformed colonies were picked onto fresh ampicillin plates and grown overnight at 37°C. Colonies were transferred to Whatman 540 filter paper and fixed (Gergen, J.P., Stern, R.H., and Wensink, P.C. (1979), Nucl.Acids Res. 7, 2115 - 2136). A prehybridization was performed in a sealed plastic bag with 6 ml of 0.9 M NaCl, 0.09 M Tris-HCl pH 7.5 0.006 M EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1% SDS and 50 µg/ml salmon sperm DNA for 2 hours at 65°C. Then 8.5 x 10^6 cpm of ^{32}P-labelled 19-mer was added and hybridization performed at 45°C overnight. The filter was washed with 0.9 M NaCl, 0.09 M sodium citrate three times at 0°C for 5 min. and was then autoradiographed and washed once at 45°C for 1 min. and autoradiographed again. After washing at 45°C, identification of 3 colonies containing mutated plasmid was possible.

Endonuclease analysis of mutated plasmids

Plasmids from the supposed mutant colonies were prepared by a rapid method (Ish-Horowicz, D. and Burke, J.F. (1981), Nucl.Acids Res. 9, 2989 - 2998), digested with a mixture of BamHI and Hind III and then analysed by electrophoresis on a 2% agarose gel. The presence of a 179 bp fragment confirmed that the 3 colonies contained mutant plasmid.

Retransformation

The colonies identified as "mutant" contain plasmids which are the progeny of a heteroduplex. Pure mutant could be obtained by retransformation of $CaCl_2$ treated E. coli MC1000 (r^-, m^+) with plasmid from 2 of the mutant colonies. From each plate 5 ampicillin resistant clones were isolated, plasmid DNA was prepared and analysed by endonuclease cleavage as mentioned above. 3 out of 5 and 5 out of 5 respectively were shown to be pure mutant. One plasmid pMT319 was selected for further use.

DNA sequence analysis

5 µg of pMT319 was cleaved with BamHI under standard conditions, phenol extracted and ethanol precipitated. Filling in of the BamHI sticky ends was performed with Klenow DNA polymerase I, dCTP, dGTP, dTTP, and α-^{32}P-dATP.

After phenol extraction and ethanol precipitation the DNA was digested with EcoRI. The ^{32}-P labelled fragment with the deletion was purified by electrophoresis on a 2% agarose gel and sequenced by the Maxam-Gilbert method (Maxam, A. and Gilbert, W. (1980) Methods in Enzymology 65, 499 - 560).

Example 2

Construction of a yeast plasmid pMT344 for expression of B(1-29)-A(1-21) of human insulin (B'A).

Plasmid pMT319 containing the gene coding for B'A and constructed as explained above was cut with restriction enzymes Hind III and XbaI and a 0.18 kb fragment was isolated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold

Spring Harbor Press 1982) from a 2% agarose gel. Similarly a fragment (6.5 kb XhoI - Hind III) containing the S. cerevisiae TPI promotor (TPI_p) (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae, J.Mol. Applied Genet. 1 (1982) 419 - 434) and the MFα1 leader sequence (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) was isolated from plasmid p285 constructed as described in US-patent application S.N. 547,748 of November 1, 1983. P285 contains the insert TPI_p-MFα1 leader -B-C-A- TPI_T and was deposited in yeast strain Z33 (ATCC No. 20681). A fragment (0.7 kb XbaI - BamHI) containing the TPI transcription termination sequences (TPI_T) (T. Alber and G. Kawasaki, Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434) was also isolated from p285. Finally a 5.4 kb XhoI - BamHI fragment was isolated from the yeast vector YEp13 (J.R. Broach. Construction of High Copy Yeast Vectors Using 2μm Circle Sequences. Methods Enzymology 101 (1983) 307 - 325). The above four fragments were ligated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) and transformed into E. coli (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) selecting for ampicillin resistance. Plasmids were isolated from the transformants and the structure of one of these, pMT344, verified by restriction mapping. The construction and main features of pMT344 are outlined in fig. 1.

Example 3

Construction of a yeast plasmid pMT475 for expression of B(1-29)-A(1-21) of human insulin (B'A) after a modified MFα1 leader.

To construct a plasmid for the expression of B'A after a MFα1 leader (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943)

lacking its last four amino acids (Glu-Ala-Glu-Ala), the 0.14 kb XbaI - EcoRII fragment containing the A and part of the B' sequences was isolated from pMT319. Likewise the 5' proximal part of the B' gene was isolated as a 0.36 kb EcoRI - EcoRII fragment from pM215. Plasmid pM215 was constructed by subcloning the EcoRI - XbaI fragment containing the proinsulin B-C-A gene from p285 into pUC13 (constructed as described for pUC8 and pUC9 by Vieira et al., Gene 19: 259 - 268 (1982)) and subsequent in vitro loop-out removal of the 12 bases coding for Glu-Ala-Glu-Ala at the junction between MFα1 leader and proinsulin B-C-A gene. These two pieces covering the B'A gene were ligated to EcoRI - XbaI digested pUC13 vector (see fig. 2) to give pMT473. The modified gene contained within a 0.5 kb EcoRI - XbaI fragment was isolated from pMT473 and then ligated to two fragments (4.3 kb XbaI - EcoRV and 3.3 kb EcoRV - EcoRI) from pMT342. pMT342 is the yeast vector pMT212 with an inserted TPI_P-MFα1 leader - B-C-A - TPI_T. The resulting plasmid, pMT475, contains the insert: TPI_P - MFα1 leader (minus Glu-Ala-Glu-Ala) - B'A - TPI_T. The construction of plasmids pMT342, pMT473 and pMT475 is outlined in fig. 2. The construction of the vector pMT212 is shown in fig. 3. Plasmid pMLB1034 is described by M.L. Berman et al., Advanced Bacterial Genetics, Cold Spring Harbor (1982), 49 - 51 and pUC12 was constructed as described for pUC13 (Vieira et al, ibid.).

Example 4

Insertion of the B(1-29)-A(1-21) (B'A) gene into a stable yeast plasmid pMT479.

The modified B'A gene from pMT475 was isolated as a 2.1 kb BamHI - partial SphI fragment and ligated to an approximately 11 kb BamHI - SphI fragment of plasmid CPOT (ATCC No. 39685) to give plasmid pMT479 (fig. 4). Plasmid CPOT is based on the vector C1/1 which has been modified by substituting the original pBR322 BglI - BamHI fragment with the similar BglI - BamHI fragment from pUC13 and subsequent insertion of the S.pombe TPI gene (POT) (US patent application S.N. 614,734 filed on May 25, 1984) as a BamHI

- SalI fragment to give CPOT. Cl/1 is derived from pJDB 248, Beggs et al., Nature 275, 104 - 109 (1978) as described in EP patent application 0103409A.

Example 5

Transformation

S. cerevisae strain MT118 (a, leu 2, ura 3, trp 1) was grown on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) to an OD_{600} of 2.1. 100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of (1.2 M sorbitol, 25 mM Na_2EDTA pH= 8.0, 6.7 mg/ml dithiotreitol). The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of (1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate pH = 5.8, 2 mg Novozym® 234 enzyme). The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and in 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (Tris = Tris(hydroxymethyl)-aminometan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of plasmid pMT344 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) with leucine omitted and containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked

after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant MT350 (=MT 118/pMT344) was chosen for further characterization.

Plasmid pMT475 was transformed into S.cerevisiae strain MT 362 (α,leu2) by the same procedure as above, and the transformant MT371 (=MT362/pMT475) isolated.

Transformation of pMT479 into strain E2-7B X E11-3C (a/α, Δtpi/Δtpi, pep 4-3/pep 4-3; this strain will be referred to as MT501) was performed as above with the following modifications: 1) prior to transformation strain MT501 was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an OD_{600} of 0.6. 2) the SOS solution contained YPGaL instead of YPD. One transformant MT519 (=MT501/pMT479) was chosen for further characterization.

The transformed microorganisms MT 350, MT 371 and MT 519 were deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), Griesebachstrasse 8, D-3400 Göttingen, on May 15, 1984 and accorded the reference numbers DSM 2957, DSM 2958, and DSM 2959, respectively.

Example 6

Expression of B(1-29)-A(1-21) insulin in yeast

Strains MT350 (DSM 2957) and MT371 (DSM 2958) were grown in synthetic complete medium SC (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981) with leucine omitted. For each strain, two 1 liter cultures in 2 liter baffled flasks were shaken at 30°C until they reached OD_{600nm} of 7 to 10. They were then centrifuged and the supernatant removed for further analysis.

Strain MT519 (DSM 2959) was grown similarly but on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) and to an OD_{600nm} of 15, centrifuged and the supernatant separated for analysis as above.

Example 7

Expression of B(1-29)-A(1-21) insulin in yeast strain MT350 (DSM 2957)

Yeast strain MT350 (DSM 2957) was grown as previously described in example 6 and expression products from 1100 ml of supernatant from this strain were isolated as follows:

10 g of LiChroprep® RP-18 (Merck, art. 9303) were washed 3 times with 50 mM NH_4HCO_3, 60% EtOH and thereafter packed in a 6 x 1 cm column. The column was equilibrated with 50 ml of 50 mM NH_4HCO_3. 55 ml of 96% EtOH were added to 1100 ml of the yeast supernatant, and the mixture was applied to the column overnight (flow: 70 ml/h).

The column was washed with 10 ml of 0.5 M NaCl and 10 ml of H_2O, and the peptides were eluted with 50 mM of NH_4HCO_3, 60% EtOH. The eluate (5 ml) was concentrated by vacuum centrifugation to 1.4 ml (to remove the ethanol), and the volume was adjusted to 10 ml with 25 mM of HEPES buffer pH = 7.4. The sample was applied to an antiinsulin immunoabsorption column (AIS column) (2.5 x 4.5 cm) which had been washed 4 times with 5 ml of NaFAM-buffer (Heding, L., Diabetologia 8, 260-66, 1972) and twice with 5 ml of 25 mM HEPES-buffer prior to the application. After the application, the column was allowed to stand for 30 min. at room temperature and was thereafter washed 10 times with 4 ml of 25 mM HEPES buffer. The peptides were eluted with 20% HAc. The pH value of the eluate was adjusted to 7.0 with NH_4OH, and the pool was concentrated to 500 µl by vacuum rotation.

The sample from the previous step was further purified on HPLC on a 10µ Waters µBondopak C-18 column (3.9 x 300 mm). The A and B buffers were 0.1% TFA in H_2O and 0.07% TFA in MeCN, respectively. The column was equilibrated with 25% B (flow: 1.5 ml/min.) and the peptides were eluted with a linear gradient of MeCN (1%/min.) and detected at 276 nm. The yield in each step of the purification was determined by radioimmunoassay as previously described, and Table 2 summarizes the purification. The overall yield was 68%.

Table 2

Purification of expression products from yeast strain MT350 supernatant

Purification step	Volume (ml)	Immunoreactive B(1-29)-A(1-21) insulin (nmol)
Supernatant	1100	110[x]
RP-18	10	116
Anti-insulin Sepharose	0.5	116
HPLC	2.5	75

x) Dilution effect was observed in this sample

Only one peak containing immunoreactive B(1-29)-A(1-21) insulin material was detected from the HPLC column. Peptide material from this peak was isolated and subjected to amino acid sequence analysis. The sequence analysis was performed with a Gas Phase sequencer (Applied Biosystem Model 470A) as described by Hewick, R.M. et al. (J.Biol.Chem. 256, 7990-7997, 1981). From the sequencing results it could be concluded that the expression products consisted of 3 peptides:

$(Glu\text{-}Ala)_2$-B(1-29)-A(1-21) insulin	89%
Glu-Ala-B(1-29)-A(1-21) insulin	2%
B(1-29)-A(1-21) insulin	9%

The peptides were present in the relative amount as indicated.

Example 8

Expression of B(1-29)-A(1-21) insulin in yeast strain MT371 (DSM 2958)

Yeast strain MT371 (DSM 2958) was grown as previously described in example 6 and expression products from 665 ml of supernatant from this strain were isolated as described in Example 7. The overall yield was 50 nmol, corresponding to 39%. Peptide material was isolated from the HPLC column and sequenced

as described in Example 7. From the sequence results (18 residues from the N-terminal) it could be concluded that the peptide was homogeneous B(1-29)-A(1-21) insulin.

Comparison of these results to the results obtained in Example 7 indicates the advisability of removing the Glu-Ala-Glu-Ala sequence from the C-terminal of the MFα1 leader. It appears from Example 7 that the yeast dipeptidase enzyme does not function very efficiently in splitting off the Glu-Ala and Glu-Ala-Glu-Ala from the B(1-29)-A(1-21) insulin prior to secretion of the insulin precursor from the yeast cells.

Example 9

Expression of B(1-29)-A(1-21) insulin in yeast strain MT519 (DSM 2959)

Yeast strain MT519 (DSM 2959) was grown as previously described in example 6 and expression products from 70 ml of supernatant were isolated as described in example 7. The overall yield was 116 nmol, corresponding to 57%. The peptide was sequenced as described in Example 7. As judged from the 42 residues identified from the N-terminal end, the peptide was homogeneous B(1-29)-A(1-21) insulin. Approximately 5 nmol of peptide was hydrolyzed in 100 µl 6N HCl for 24 h at 110°C. The hydrolysate was analyzed on a Beckman Model 121M amino acid analyser. The following amino acid composition was found:

Table 3

Amino acid analysis of purified B(1-29)-A(1-21) insulin

Amino acid	Found	Theoret.	Amino acid	Found	Theoret.
Asx*	2.97	3	Val	3.37	4
Thr	1.77	2	Ile	1.65	2
Ser	2.45	3	Leu*	5.65	6
Glx*	6.68	7	Tyr	3.51	4
Pro	1.33	1	Phe*	2.73	3
Gly*	3.95	4	Lys*	0.95	1
Ala*	1.22	1	His*	1.84	2
Cys 0.5	4.54	6	Arg*	1.13	1

*) amino acid used for normalization.

Example 10

Construction of a yeast plasmid pMT610 for expression of B(1-29)-Ala-Ala-Lys-A(1-21)

A 4.3 kb EcoRV-XbaI and a 3.3 kb EcoRI-EcoRV fragment from pMT342 (see example 3) were ligated to a 0.6 kb EcoRI-XbaI fragment of pM215 (see example 3). The obtained plasmid pMT462 harbours the insert MFα1 leader (minus Glu-Ala-Glu-Ala)-B-C-A. For converting the B-C-A encoding fragment into a B(1-29)-Ala-Ala-Lys-A(1-21) encoding fragment the modified site specific mutagenesis procedure (K. Norris et al., ibid.) was used. A 0.6 kb EcoRI-XbaI fragment from pMT462 encoding MFα1 leader (minus Glu-Ala-Glu-Ala)-B-C-A was inserted into M13 mp10 RF phage cut with XbaI-EcoRI. Single strand M13 phage containing the above EcoRI-XbaI insert was incubated with a 30mer d(TTCACAATGCCCTTAGCGGCCTTGGGTGTG) primer (KFN15) and the "universal" 15-mer M13 primer d(TCCCAGTCACGACGT) (see example 1), heated to 90°C for 5 minutes and slowly cooled to room temperature in order to allow annealing. Then partly double stranded DNA was made by addition of a d-NTP-mix, Klenow Polymerase and T4 ligase.After phenol extraction, ethanol precipitation and resuspension, the DNA was cut with restriction enzymes ApaI,XbaI and EcoR1. After another phenol extraction, ethanol precipitation and resuspension, the DNA was ligated to EcoR1-XbaI cut pUC13. The ligation mixture was transformed into an E.coli (r^-m^+) strain and plasmids were prepared from a number of transformants. Plasmid preparations were cut with EcoR1 and XbaI and those preparations showing bands at both 0.5 and 0.6 kb were retransformed into E.coli. From the retransformation a transformant harbouring only pUC13 with a 0.5 kb insert was selected. The sequence of the EcoR1-XbaI insert of this plasmid, pMT598, was then confirmed by the Maxam-Gilbert method to encode MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21). The XbaI-EcoRI insert from pMT598 was provided with TPI promotor and TPI terminator by ligation of a 0.5 kb XbaI-EcoRI fragment of pMT598 with a 5.5 kb XbaI-EcoRI fragment of pT5. The construction

of pT5 harbouring the insert TPIp-MFα1 leader-B-C-A-TPI_T is illustrated in fig. 8. The resulting plasmid pMT 601 containing the insert TPIp-MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21)-TPI_T was cut with BamH1 and partially with Sphl and the 2.1 kb fragment was inserted in CPOT cut with BamHI and Sphl. The resulting plasmid pMT610 was used for transformation of yeast.

Example 11

Construction of a yeast plasmid pMT639 for expression of B(1-29)-Ser-Lys-A(1-21)

The BCA encoding fragment from pMT462 (see example 10) was converted into B(1-29)-Ser-Lys-A(1-21) by a procedure analogous with the procedure described in example 10 by site specific mutagenesis with a mixture of a 27-mer d(TCCACAATGCCCTTAGACTTGGGTGTG) primer KFN36 and the "universal" 15-mer M13 primer. After filling in with Klenow polymerase and ligation with T4 ligase the partly double stranded DNA was digested with ApaI, EcoRI and XbaI and ligated with the 5.5 kb XbaI - EcoRI fragment from plasmid pT5 (see example 10). After transformation and retransformation into E.coli, a plasmid pMT 630 containing the insert MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21) was isolated and the sequence of the insert confirmed. The further procedure for obtaining plasmid pMT639 containing the insert TPIp-MFα1 (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPI_T was as described in example 10. The construction of pMT639 is illustrated in Fig. 9.

Example 12

Expression of B(1-29)-Ala-Ala-Lys-A(1-21) in yeast strain MT 620

S. cerevisiae strain MT501 (see example 5) was transformed with pMT 610 as described for pMT479 in example 5. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant MT 620 = (MT501/pMT610) was chosen for further characterization. MT620 was deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), on January 16, 1985 and accorded the reference number DSM 3196.

MT 620 was grown on YPD medium. A two liter culture in 2 liter baffled flask was shaken at 30°C to an OD_{600nm} of 15. After centrifugation the supernatant was removed for further analysis. The expression level determined by radioimmunoassay was 1.2 µmol/l. Expression products from 840 ml of supernatant were purified as described in Example 7. (RP-18 column, Anti-insulin Sepharose and HPLC). The overall yield was 100 nmol corresponding to about 10%. Peptide material was isolated from the HPLC-column and sequenced as described in Example 7. 35 Edman degradation cycles were carried out (Table 4). From the sequence results the position of the 3 amino acid residue chains (Ala-Ala-Lys) separating the B(1-29) and the A(1-21) chains was confirmed (see table 4).

Table 4

Sequence analysis of B(1-29)-Ala-Ala-Lys-A(1-21) isolated from the culture medium of strain MT 620.

Cyclus No.	PTH-amino acid residue	Yield (pmol)
1	Phe	3381
2	Val	1738
3	Asn	5169
4	Gln	2750
5	His	2045
6	Leu	1405
7	Cys	-
8	Gly	1372
9	Ser	345
10	His	1105
11	Leu	2228
12	Val	1963
13	Glu	1219
14	Ala	1514
15	Leu	1793
16	Tyr	1707
17	Leu	1354
18	Val	1765
19	Cys	-
20	Gly	882
21	Glu	1019
22	Arg	1100
23	Gly	1123
24	Phe	1492
25	Phe	2042
26	Tyr	1014
27	Thr	195
28	Pro	710
29	B_{29}Lys	1173
30	Ala	1026
31	Ala	885
32	Lys	1175
33	A_1Gly	552
34	Ile	518
35	Val	548

The average repetitive yield was 95.6%.

Example 13

Expression of B(1-29)-Ser-Lys-A(1-21) in yeast strain MT643

S. cerevisiae strain MT501 was transformed with pMT639 as described for pMT479 in example 5.

One transformant MT643 = (MT501/pMT639) was chosen for further characterization. MT643 was deposited by the applicant at DSM on January 16, 1985 and accorded the reference No. DSM 3197.

MT643 was grown as described in example 12. After centrifugation the supernatant was removed for further analysis.

The expression level of the insulin precursor determined by radioimmunoassay was 1.6 µmol/l. Expression products from the supernatant from strain MT 643 was isolated as described in Example 7. The peptide material isolated from the HPLC column was submitted to sequence analysis as described in Example 7. From the sequence results (not shown) the position of the two amino acid residues chains (Ser-Lys) separating the B(1-29) and A(1-21) chains was confirmed.

Example 14

Conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10 M acetic acid. 0.26 ml of 1.54 M Thr(Bu^t)-OBu^t in N,N-dimethylacetamide was added. The mixture was cooled to 12°C. 2.8 mg of trypsin dissolved in 0.035 ml of 0.05 M calcium acetate was added. After 72 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin was 64% by HPLC.

Example 15

Conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10 M acetic acid. 0.26 ml of 1.54 M Thr-OMe in a mixture of dimethyl sulphoxide and butane-1,4 diol 1/1 (v/v) was added. 1 mg of lysyl endopeptidase from Achromobacter lyticus (Wako Pure Chemical Industries, Osaka, Japan) in 0.07 ml of water was added. After 120 hours at 25°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation, and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin was 75% by HPLC.

Example 16

Conversion of B(1-29)-Ser Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg of B(1-29)-Ser-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N-dimethylformamide (v/v) in water. 0.2 ml of 2 M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12°C. 2 mg of trypsin in 0.05 ml 0.05 M calcium acetate was added. After 24 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ser-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 85% by HPLC.

Example 17

Conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg of B(1-29)-Ala-Ala-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N dimethylformamide (v/v) in water. 0.2 ml of 2 M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12°C. 2 mg of trypsin in 0.05 ml 0.05 M calcium acetate was added. After 96 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 84% by HPLC.

Example 18

Preparation of human insulin from various human insulin esters

The human insulin esters in the crude acetone precipitates were purified by gelfiltration and anion exchange chromatography as described in Methods in Diabetes Research vol.1, p. 407 - 408 (Eds. J. Larner & S. Pohl (John Wiley Sons, New York, 1984)). The method was applicable to any of the 3 human insulin esters. The cleavages of the various ester groups, rendering human insulin in nearly 100% yields, were carried out

by hydrolysis of Thr-OMe(B30) human insulin and by acidolysis with trifluoroacetic acid of Thr(Bu^t)-OBu^t(B30) human insulin and of Thr-OBu^t(B30) human insulin as described ibid. p. 409.

CLAIMS

1. A DNA-sequence comprising a sequence encoding an insulin precursor of the formula:

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n ~~naturally occurring~~ amino acid residues, Y is Lys or Arg, n = 0 to 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain -X_n-Y- does not contain two adjacent basic amino acid residues.

2. A DNA-sequence according to claim 1 wherein m is 0.

3. A DNA-sequence according to claim 1 wherein m is 1 and n is 1-8.

4. A DNA-sequence according to claim 1 wherein said insulin precursor is

B(1-29)-Ala-Ala-Lys-A(1-21).

5. A DNA-sequence according to claim 1 wherein said insulin precursor is B(1-29)-Ser-Lys-A(1-21).

6. A replicable expression vehicle capable of expressing in yeast a DNA-sequence encoding an insulin precursor of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n ~~naturally occurring~~ amino acid residues, Y is Lys or Arg, n = 0 to 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain -X_n-Y- does not contain two adjacent basic amino acids.

7. A replicable expression vehicle according to claim 6 comprising the plasmid pMT344.

8. A replicable expression vehicle according to claim 6 comprising the plasmid pMT475.

9. A replicable expression vehicle according to claim 6 comprising the plasmid pMT479.

10. A replicable expression vehicle according to claim 6 comprising the plasmid pMT610.

11. A replicable expression vehicle according to claim 6 comprising the plasmid pMT639.

12. A yeast strain transformed with a replicable expression vehicle capable of expressing in yeast a DNa-sequence encoding an insulin precursor of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n ~~naturally occurring~~ amino acid residues, Y is Lys or Arg, n = 0 to 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain -X_n-Y- does not contain two adjacent basic amino acids.

13. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT344.

14. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT475.

15. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT479.

16. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT610.

17. A yeast strain according to claim 12, wherein said replicable expression vehicle comprises the plasmid pMT639.

18. A process for producing an insulin precursor of the formula:

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n expressible ~~naturally occurring~~ amino acid residues, Y is Lys or Arg, n = 0 to 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain -X_n-Y- does not contain two adjacent basic amino acid residues; said process comprising the steps of:

culturing a transformant yeast strain in a suitable nutrient medium, said transformant yeast strain including a replicable expression vehicle capable of expressing a DNA-sequence encoding said insulin precursor, and recovering said insulin precursor.

19. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 2957 ~~or a variant or mutant thereof~~.

20. A process for producing an insulin precursor according to claim 19, wherein said insulin precursor has the formula B(1-29)-A(1-21).

21. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 2958 ~~or a variant or mutant thereof~~.

22. A process for producing an insulin precursor according to claim 21, wherein said insulin precursor according to claim 21, wherein said insulin precursor has the formula B(1-29)-A(1-21).

23. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 2959 ~~or a variant or mutant thereof~~.

24. A process for producing an insulin precursor according to claim 23, wherein said insulin precursor has the formula B(1-29)-A(1-21).

25. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 3196 ~~or a variant or mutant thereof~~.

26. A process for producing an insulin precursor according to claim 25, wherein said insulin precursor has the formula B(1-29)-Ala-Ala-Lys-A(1-21).

27. A process for producing an insulin precursor according to claim 18, wherein said yeast strain is DSM 3197 ~~or a variant or mutant thereof~~.

28. A process for producing an insulin precursor according to claim 27, wherein said insulin precursor has the formula B(1-29)-Ser-Lys-A(1-21).

29. A process for preparing human insulin comprising the steps of:

(P) transforming a yeast strain with a replicable expression vehicle capable of expressing a DNA-sequence encoding an insulin precursor of the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21),$$

wherein X_n is a peptide chain with n ~~naturally occurring~~ expressible amino acid residues, Y is Lys or Arg, n = 0 to 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the chain $-X_n-Y-$ does not contain two adjacent basic amino acids;

culturing said transformed yeast strain in a suitable nutrient medium so that expression of said insulin precursor occurs;

recovering said expressed insulin precursor from said nutrient medium; and

converting said insulin precursor into human insulin.

30. Human insulin precursors of the general formula

B(1-29)-X_n-Y-A(1-21),

wherein X_n is a peptide chain with n ~~naturally occurring~~ expressible amino acid residues, n = 0 to 33, Y is Lys or Arg, B(1-21) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29}, A(1-21) is the A-chain of human insulin, and the peptide chain $-X_n-Y-$ does not contain two adjacent, basic amino acid residues.

31. A human insulin precursor according to claim 30 having the formula

B(1-29)-Ala-Ala-Lys-A(1-21).

32. A human insulin precursor according to claim 30 having the formula

B(1-29)-Ser-Lys-A(1-21).

Declaration for Patent Application and Power of Attorney

Docket No. 104-MF-2196 2620.010-DK

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled DNA-sequence Encoding Biosynthetic Insulin Precursors and Process for Preparing the Insulin Precursors and Human Insulin, the specification of which

(check one) [X] is attached hereto
[ ] was filed on _____ as Serial No. _____
and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed	
2665/84	DENMARK	30 May 84	X	
582/85	Denmark	8 February 85	X	
(Number	(Country	(Day/Month/Year)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)
(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Morris Fidelman, Reg.No.17,126; Franklin D. Wolffe, Reg.No.19,724; James S. Waldron, Reg. No. 24,650 Address all telephone calls to Fidelman,Wolffe & Waldron Telephone No. 833-8801 Address correspondence to 2120 L Street, N.W. Wash. D. C. 20037

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor JAN MARKUSSEN
Inventor's signature Jan Markussen Date May 17, 1985
Residence 7, Kikudbakken
Citizenship Danish
Post Office Address DK-2730 Herlev, Denmark

Full name of second joint inventor, if any NIELS FIIL
~~Second~~ Inventor's Signature Niels Fiil Date May 17, 1985
Residence 5, Fuglebakkevej
Citizenship Danish
Post Office Address DK-2000 Copenhagen F, Denmark

Full name of third joint inventor, if any Mogens Trier Hansen
~~Third~~ Inventor's Signature Mogens Trier Hansen Date May 6, 1985
Residence 21, Vinkelvej
Citizenship Danish
Post Office Address DK-3650 Ølstykke, Denmark

Declaration for Patent Application and Power of Attorney

Docket No. _____

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

_____, the specification of which

(check one) ☐ is attached hereto
☐ was filed on _____ as Serial No. _____
and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

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Prior Foreign Application(s):			Priority Claimed	
(Number	(Country	(Day/Month/Year)	Yes	No

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(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)
(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
Morris Fidelman, Reg.No.17,126; Franklin D. Wolffe, Reg.No.19,724; James S. Waldron, Reg. No. 24,650
Address all telephone calls to Fidelman,Wolffe & Waldron
Telephone No. 833-8801 Address correspondence to 2120 L Street, N.W. Wash. D. C. 20037

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or ~~first~~ fourth inventor Kjeld Norris
Inventor's signature _____ Date May 6, 1985
Residence 18,Eskemosegårdsalle
Citizenship Danish
Post Office Address DK-3460 Birkerød, Denmark

Full name of ~~second~~ fifth joint inventor, if any Gustav Ammerer
~~Second~~ Inventor's Signature _____ Date May 23, 1985
Residence 2617 Boyer
Citizenship Austrian
Post Office Address East Seattle, Washington 98112, USA

Full name of ~~third~~ sixth joint inventor, if any Lars Thim
~~Third~~ Inventor's Signature Lars Thim Date May 17, 1985
Residence 22, Skiftevej
Citizenship Danish
Post Office Address DK-2820 Gentofte, Denmark

Declaration for Patent Application and Power of Attorney

Docket No. _____

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

_____, the specification of which

(check one) ☐ is attached hereto
☐ was filed on _____ as Serial No. _____
and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed	
(Number	(Country	(Day/Month/Year)	Yes	No

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(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)
(Country)	(Appln. Ser. No.)	(Filing Date)	(patented, pending, abandoned)

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Morris Fidelman, Reg.No.17,126; Franklin D. Wolffe, Reg.No.19,724; James S. Waldron, Reg. No. 24,650
Address all telephone calls to Fidelman,Wolffe & Waldron
Telephone No. 833-8801 Address correspondence to 2120 L Street, N.W. Wash. D. C. 20037

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Full name of sole or ~~first~~ seventh inventor Hans Ole Voigt
Inventor's signature Hans Ole Voigt Date May 17, 1985
Residence 36, Hvidegårdsparken
Citizenship Danish
Post Office Address DK-2800 Lyngby, Denmark

Full name of second joint inventor, if any _____
Second Inventor's Signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of third joint inventor, if any _____
Third Inventor's Signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

FIG. 1

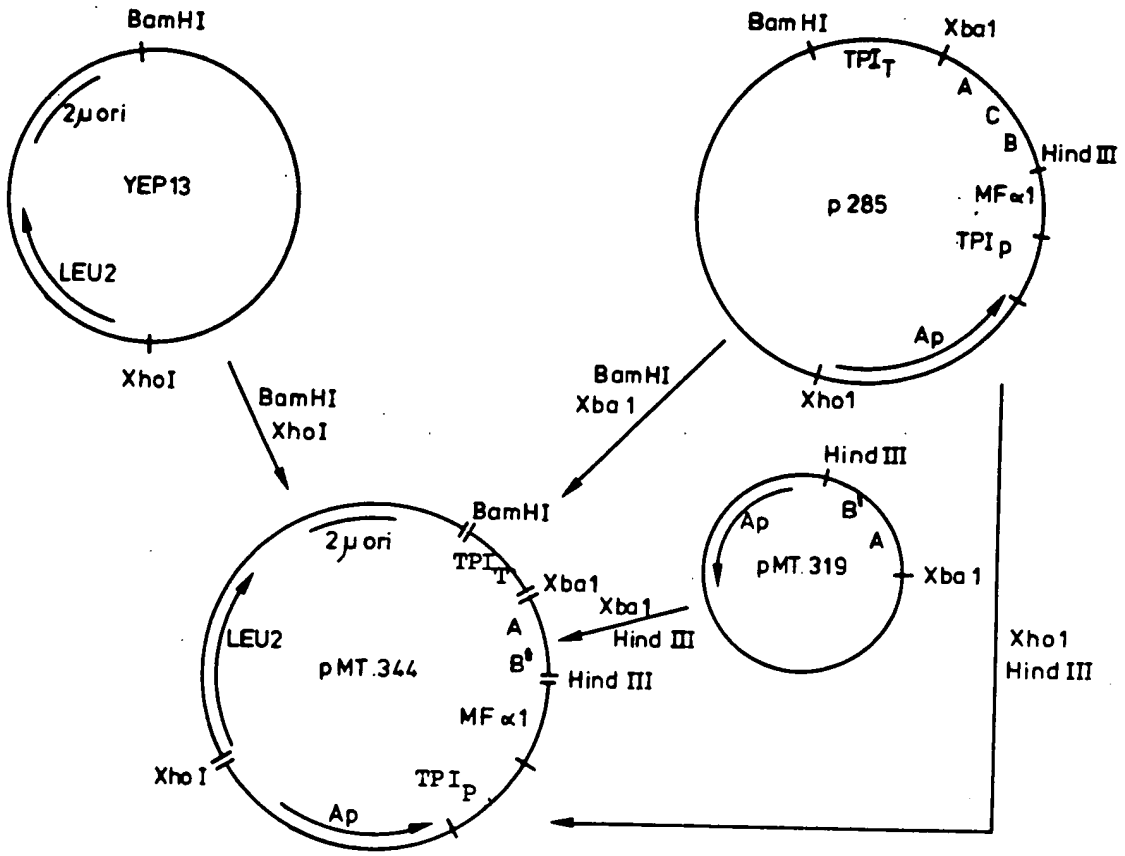

FIG. 2

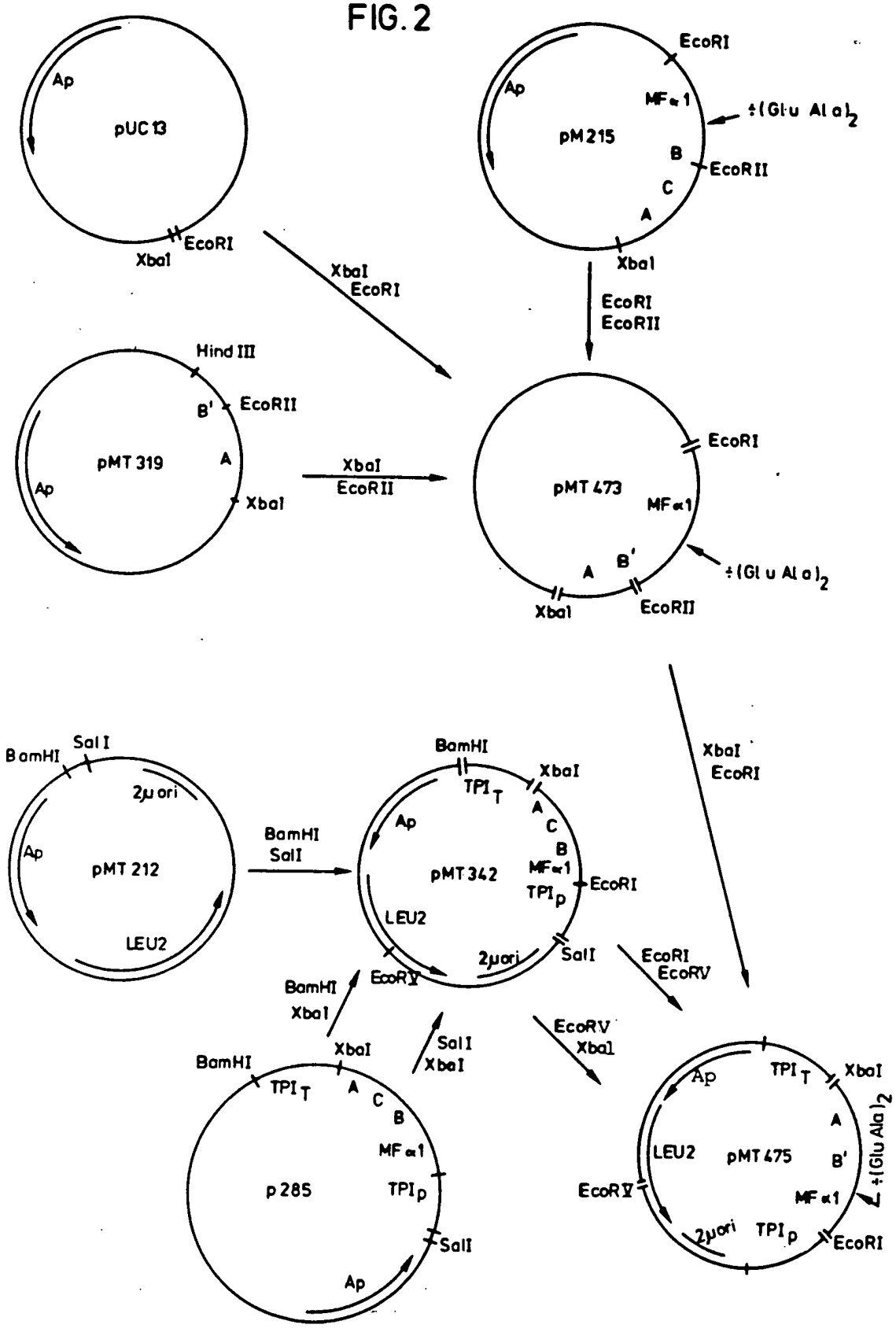

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FIG.3

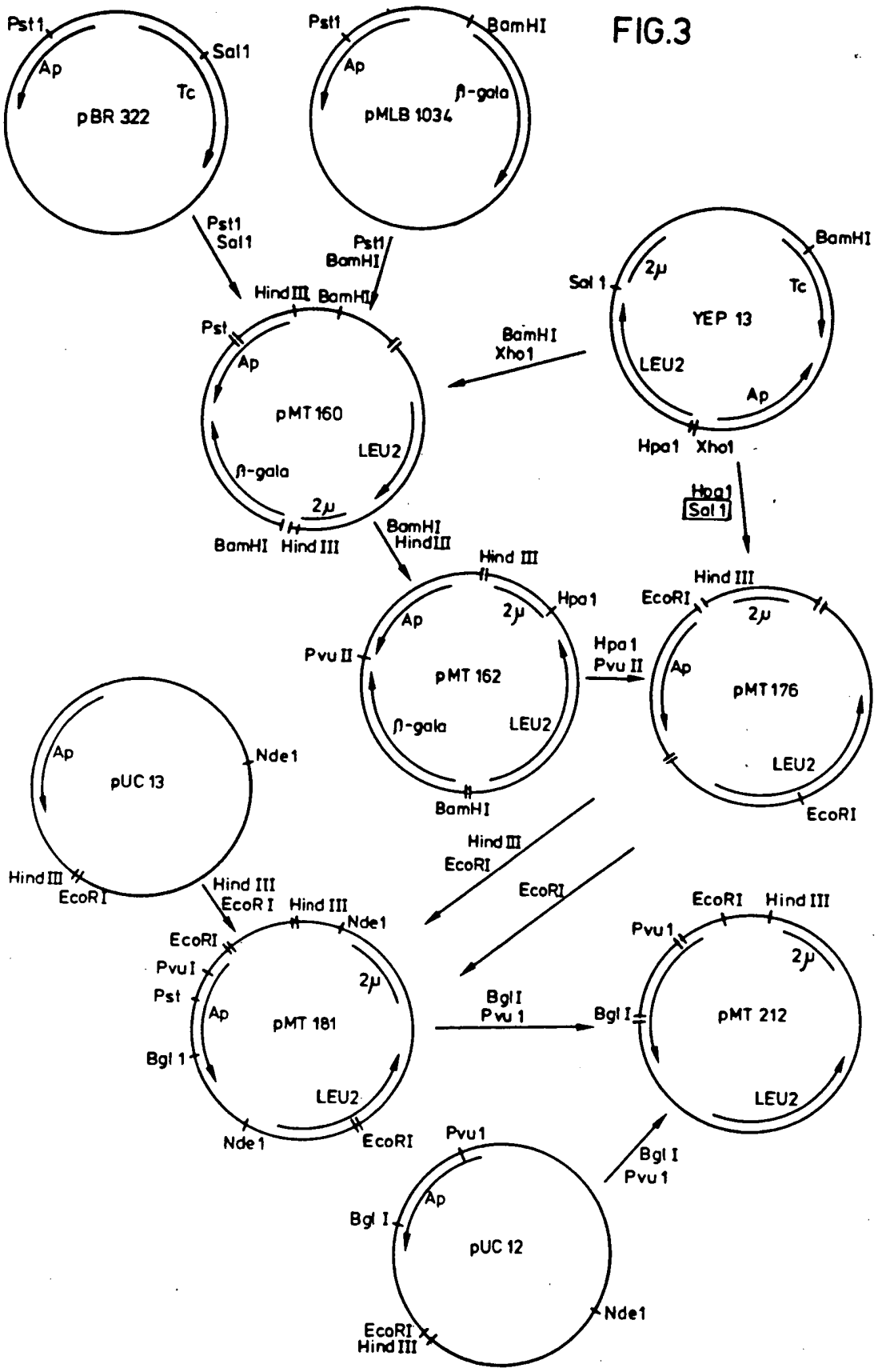


FIG. 4

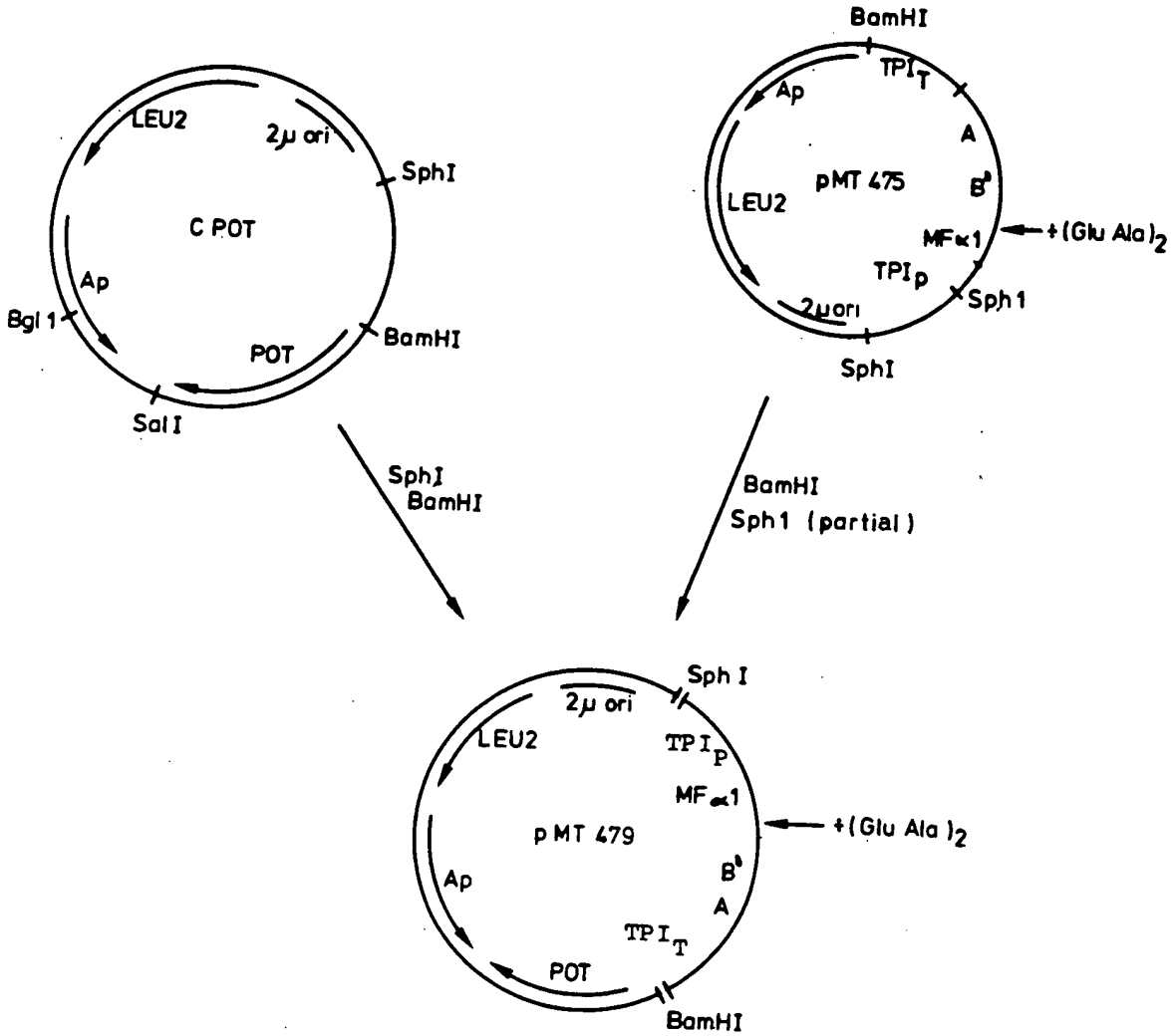


FIG. 5

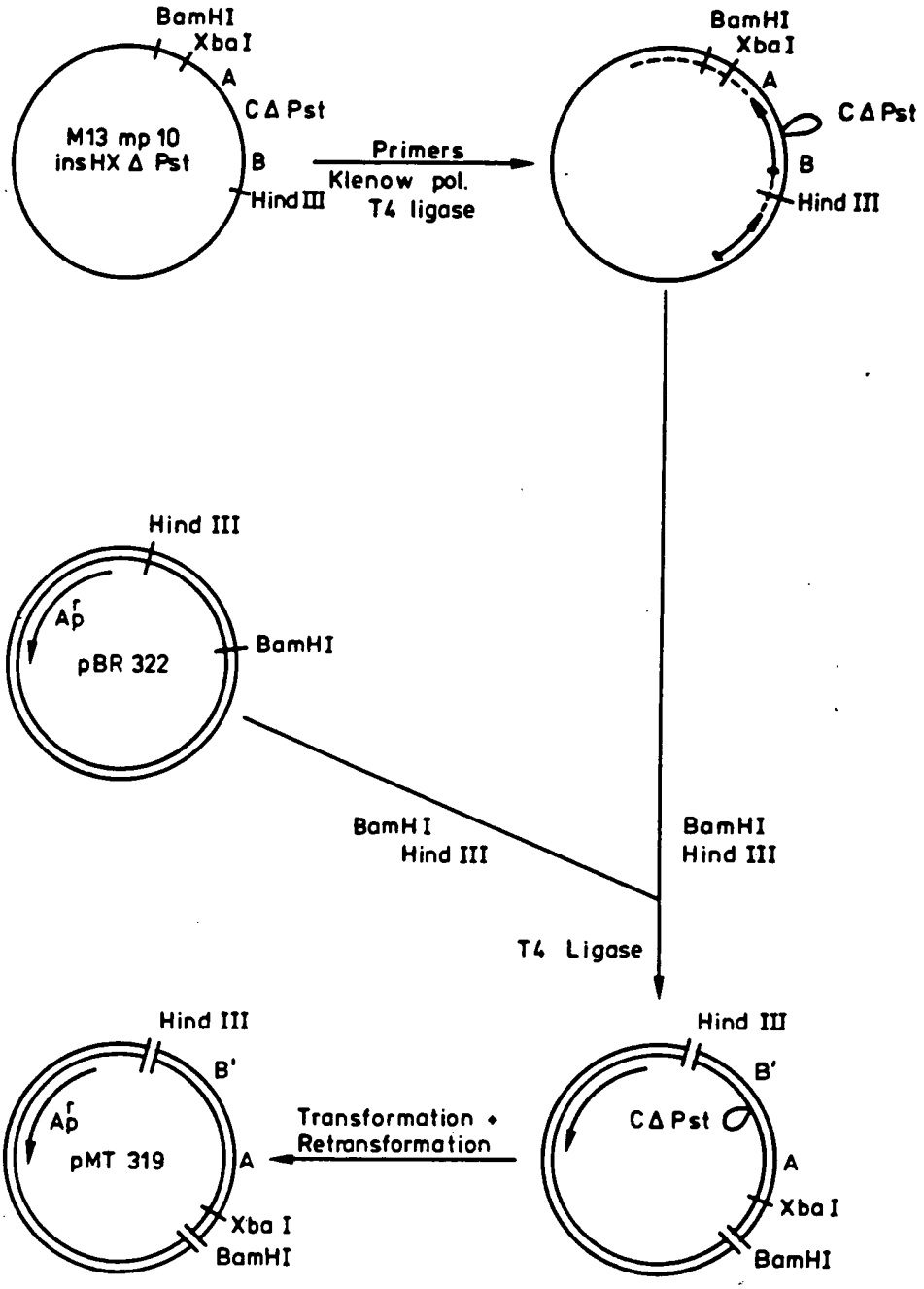


FIG.6

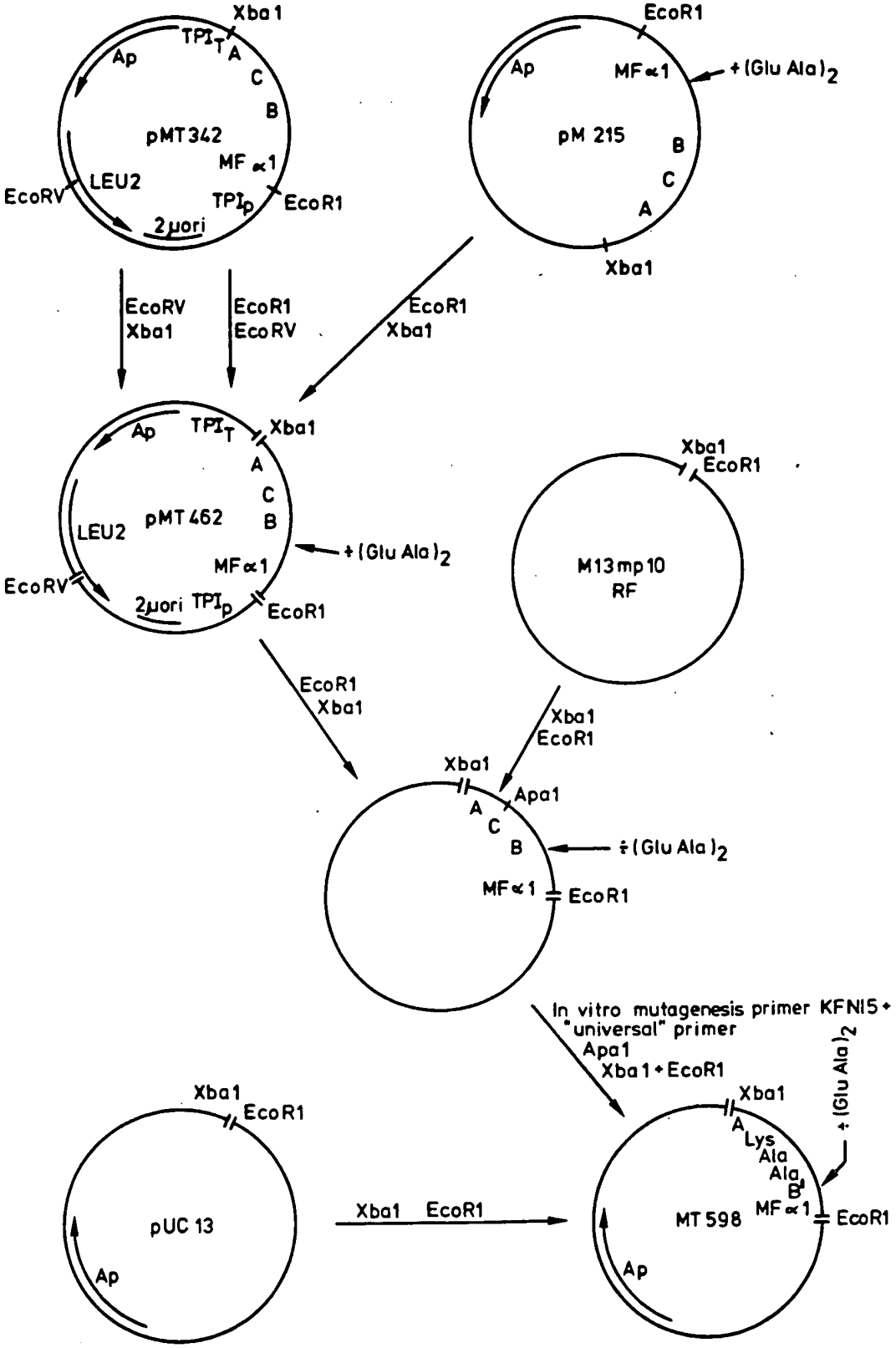


FIG. 7

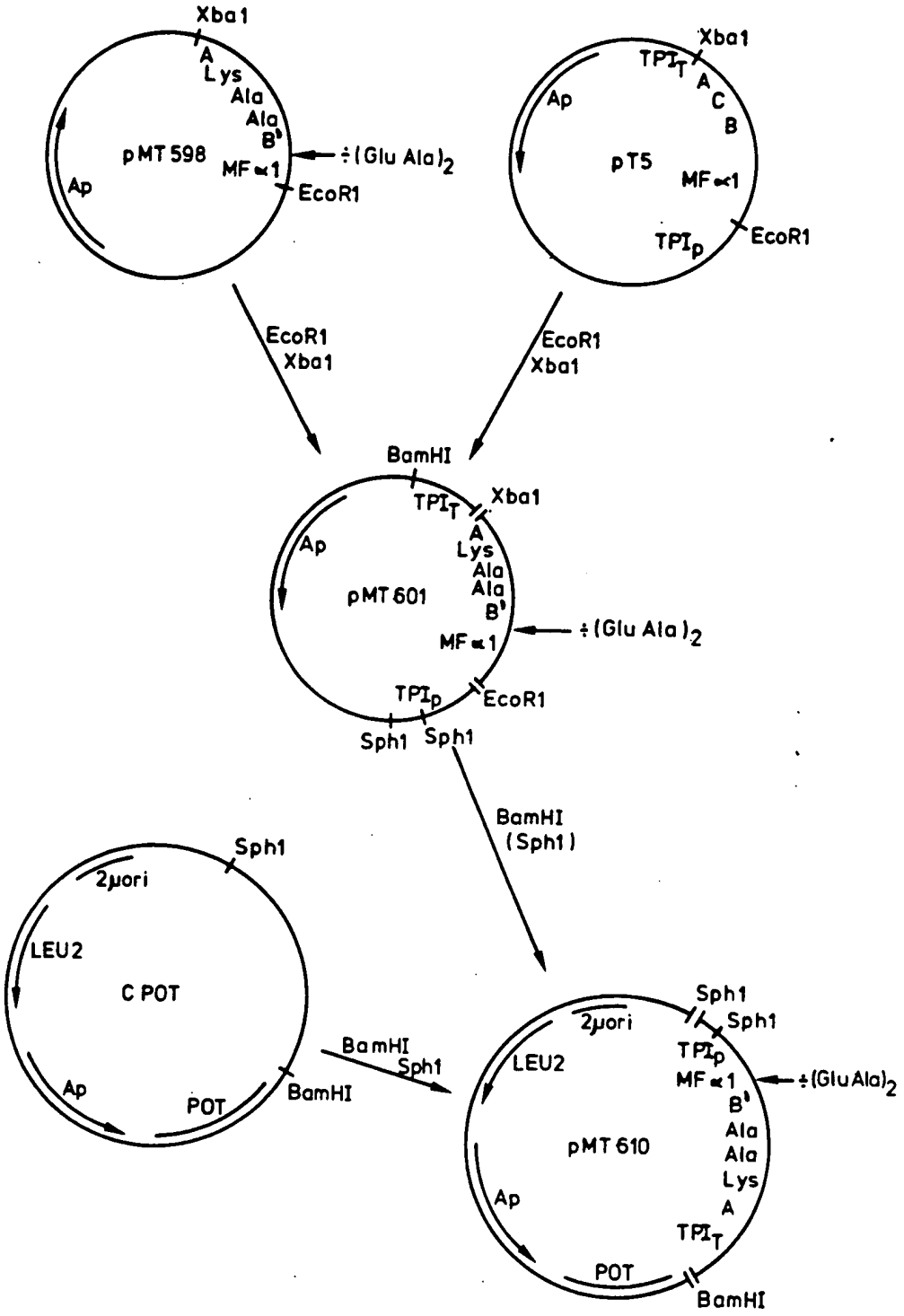


FIG. 8

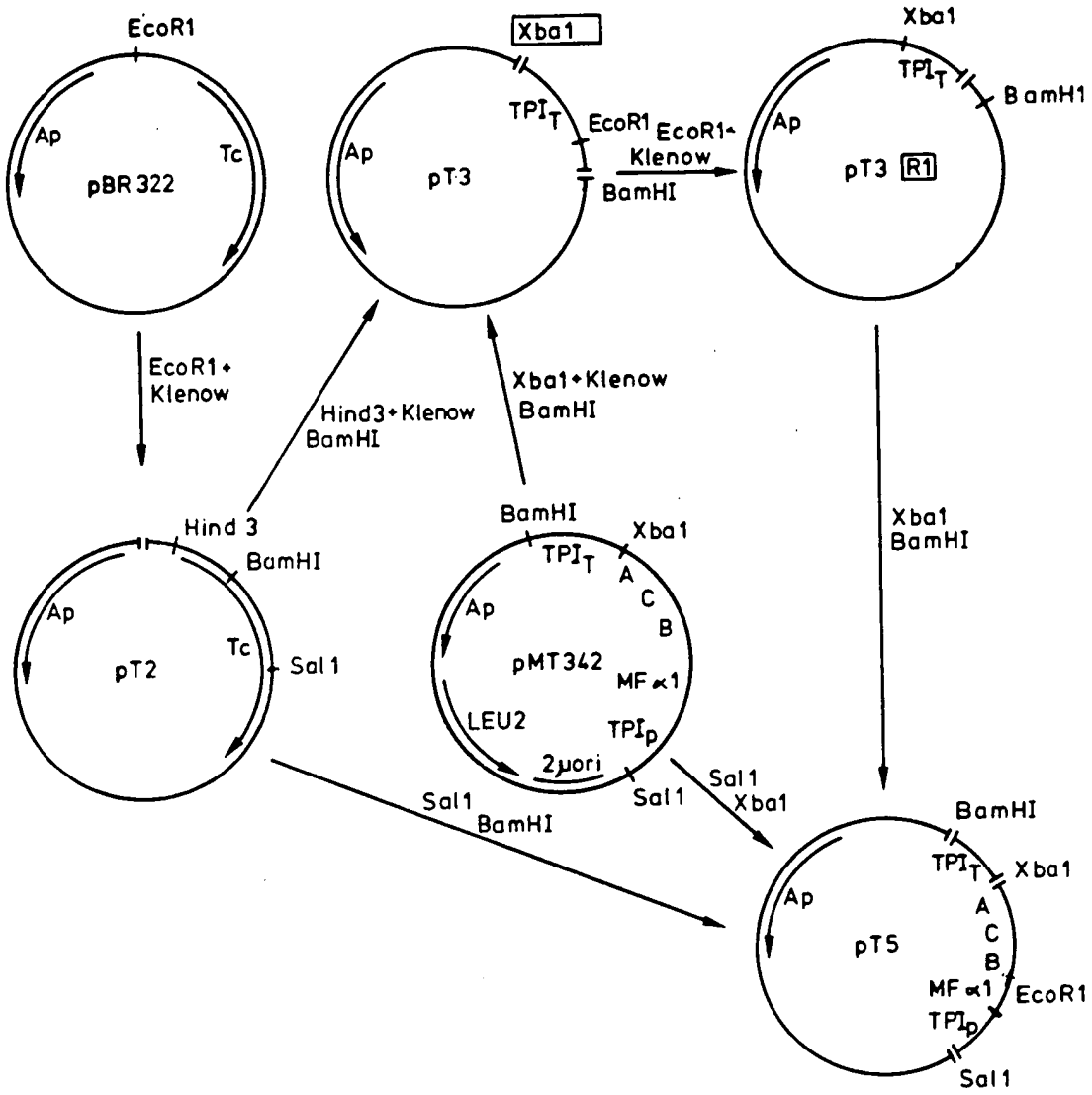
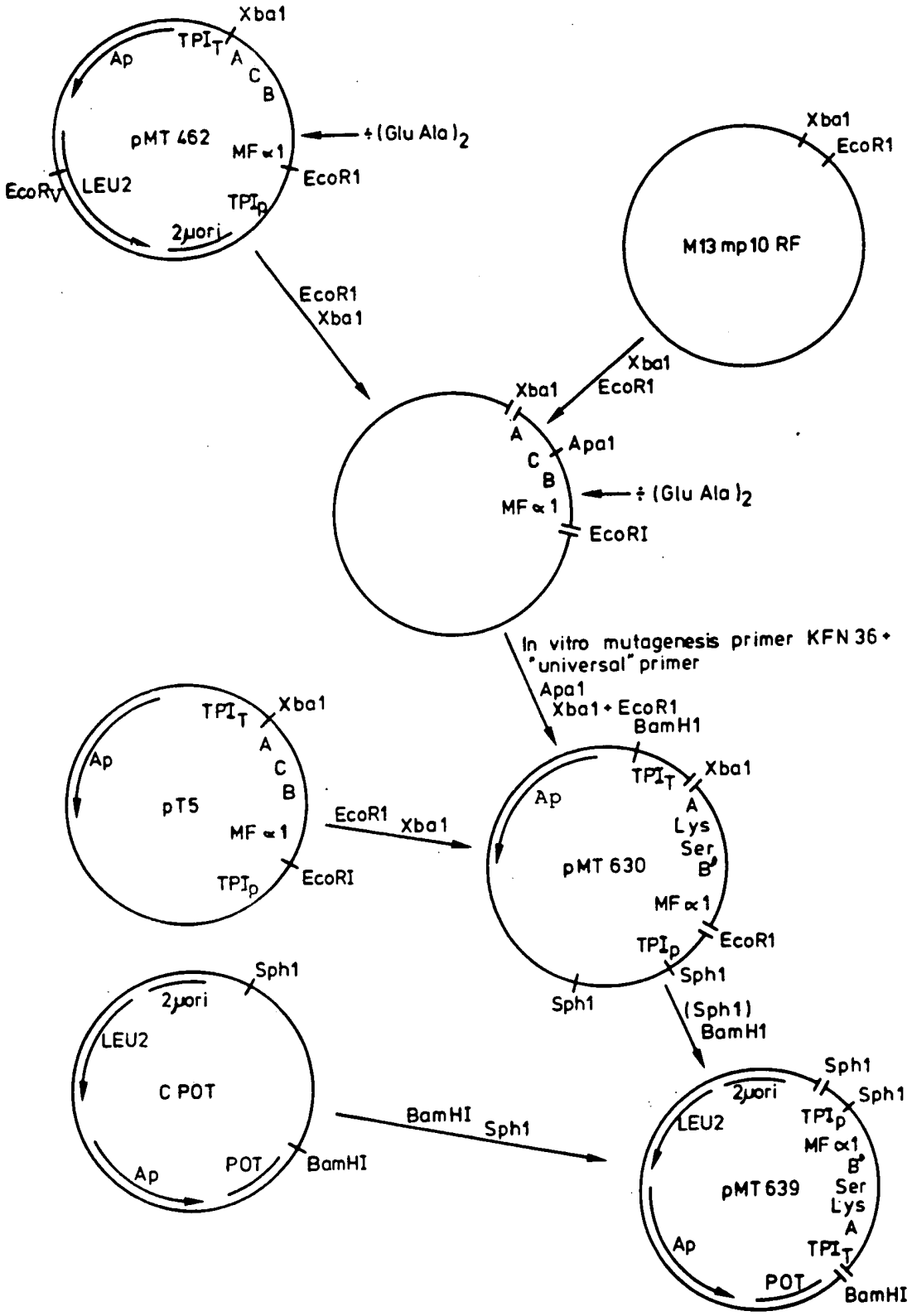


FIG. 9



#2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

JAN MARKUSSEN et al.

Filed Concurrently Herewith

For: **DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN**

CLAIM FOR PRIORITY

Honorable Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

Applicants herewith submit Certified Copies of DENMARK No. 2665/84, filed 30 May 1984, and DENMARK 582/85, filed 8 February 1985, for which a Claim of Priority under 35 U.S.C. 119 is made herein.

Respectfully Submitted,

Morris Fidelman
Reg. No. 17,126

Fidelman, Wolffe & Waldron
2120 L Street, N. W.
Suite 300
Washington, D. C. 20037
(202) 833-8801
Dated: 5/29/85
MF:js

03

039,123#2

KONGERIGET DANMARK

DIREKTORATET FOR PATENT- OG VAREMÆRKEVÆSENET

does hereby certify that on the 30th May, 1984, NOVO INDUSTRI A/S, Bagsværd, Denmark, has filed an application for a patent in Denmark for "DNA-sequence encoding a biosyntetic insulin precursor and process for preparing the insulin precursor".

(Application No. 2665 of 1984. Int. Cl. C 12 N).

The attached photocopy is a true copy of the specification, abstract and 5 drawings filed with the application.

København, den 26th April, 1985

For the D.P.V.
by order

Minna Gram

Minna Gram
Senior Clerk.

2620.000-DK
1984.05.24

ATS/KHaa
(ats 3)

DNA-sequence Encoding a Biosynthetic Insulin Precursor and Process for Preparing the Insulin Precursor

This invention relates to biosynthetic insulin. More specifically, the invention is directed to a DNA-sequence encoding a biosynthetic insulin precursor and to the preparation of such insulin precursor which is convertible into biosynthetic insulin by a transpeptidation process.

BACKGROUND OF THE INVENTION

In the past insulin has been synthezised (from synthetic A- and B-chains) or re-synthesized (from naturally derived A- and B-chains) by combining the two chains in an oxidation process whereby the 6 cysteine sulfhydryl groups of the reduced chains (4 in the A-chain, 2 in the B-chain) are converted into disulfide bonds. By this method disulfide bonds are formed largely at random, meaning that the yield of insulin with disulfide bridges correctly positioned between cysteine residues A-6 and A-11, A-7 and B-7, and A-20 and B-19, respectively, is very low.

Following the discovery of proinsulin as a biological precursor of insulin it was observed that the A- and B-polypeptide moieties of the linear-chain totally reduced proinsulin (those moieties corresponding to the A- and B-chains of insulin, respectively) could be oxidatively combined with much less randomization of the disulfide bonds to give a substantially higher yield of correctly folded proinsulin as compared with the combination of free A- and B-chains (D.F. Steiner et al.: Proc.Nat.Acad.Sci. 60 (1968), 622). Albeit high yields were obtained only at proinsulin concentrations too low to make the process feasible on a preparative scale, the function of the C- (i.e. connecting peptide) moiety of the B-C-A polypeptide sequence of proinsulin, namely that of bringing the 6 cysteine residues into spatial positions favorable for correct oxidation into proinsulin, was clearly demonstrated.

The proinsulin formed may function as an *in vitro* precursor of insulin in that the connecting peptide is removable by enzymatic means (W. Kemmler *et al.*: J.Biol.Chem. 246 (1971), 6786).

Subsequently it has been shown that proinsulin-like compounds having shorter linking moieties than the C-peptide and flanked at both ends by specific enzymatic or chemical cleavage sites (the so-called miniproinsulins (A. Wollmer et al., Hoppe-Seyler's Z. Physiol.Chem. 355 (1974), 1471 - 1476 and Dietrich Brandenburg et al., Hoppe-Seyler's Z. Physiol.Chem. 354 (1973), 1521 - 1524)) may also serve as insulin precursors.

Endeavours to provide biosynthetic insulins, particularly that identical to the human species, have followed the same strategic pathways as those to synthetic insulin. The insulin A- and B-chains have been expressed in separate host organisms, isolated therefrom and then combined as described *supra* (R.E. Chance *et al.*: Diabetes Care 4 (1982), 147). Microorganisms have been transformed with cloning vectors encoding preproinsulin or proinsulin which may be secreted as such (W. Gilbert *et al.*: European Patent Publ. No. 6694) or accumulated intracellularly as hybrid gene products (D.V. Goeddel *et al.*: European Patent Publ. No. 55945). The miniproinsulin pathway has also been attempted (D.V. Goeddel, *supra*).

Procuring the A- and B-chains in separate fermentation processes followed by combination of the chains is inherently impractical. The dual fermentation inconvenience may be overcome by choosing the proinsulin or miniproinsulin strategy. However, the use of a proinsulin as the biosynthetic insulin precursor may entail certain disadvantages. The proinsulin, whether excreted into the fermentation liquid as such or accumulated intracellularly in the host organism, possibly as a hybrid gene product, is likely to contain substantially randomized disulfide bonds. The refolding of such "scrambled" products into correctly folded proinsulin may be conducted either directly (H.-G. Gattner *et al.*: Danish Patent Application No. 4523/83) or via the single chain hexa-S-sulfonate (F.B. Hill: European Patent Publ. No. 37255). The refolding

process usually entails some degree of polymerization and hence the inconvenience of using laborious purification steps during recovery.

In addition, insulin precursors of the proinsulin type are prone to undergo enzymatic degradation, either within the host cells or following its excretion into the fermentation broth. In yeast it has been shown that human proinsulin is particularly sensitive to enzymatic cleavages at the two dibasic sequences (Arg31-Arg32 and Lys64-Arg65). Apparently these cleavages occur before the establishment of the S-S bridges, resulting in the formation of C-peptide, A-chain and B-chain.

OBJECT OF THE INVENTION AND SUMMARY THEREOF

The object of the present invention is to circumvent these disadvantages by devising a biosynthetic insulin precursor which is generated largely with correctly positioned disulfide bridges between the A- and B-moieties and, furthermore, substantially more resistant to proteolytic degradation than the biosynthetic insulin precursors known heretofore.

A single chain insulin precursor consisting of a shortened insulin B-chain from Phe^{B1} to Lys^{B29} continuing into a complete A-chain from Gly^{A1} to Asn^{A21}, B(1-29)-A(1-21), is known (Jan Markussen, "Proteolytic degradation of proinsulin and of the intermediate forms",: Proceedings of the Symposium on Proinsulin, Insulin and C-Peptide, Tokushima, 12 - 14 July, 1978, Editors: S. Baba et al.). This insulin precursor B(1-29)-A(1-21) is prepared by a semisynthetic process from porcine insulin. First the insulin B(1-29) and A(1-21) chains were prepared and coupled to a linear peptide B(1-29)-A(1-21). This compound in the hexathiol form was oxidized in vitro rendering the single chain des-(B30) insulin molecule.

The present invention is based on the surprising discovery that a biosynthetic insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin, is expressed in

high yields and with correctly positioned disulfide bridges by culturing a microorganism transformed with a DNA-sequence comprising a sequence encoding this insulin precursor.

According to a first aspect of the present invention there is provided a DNA-sequence comprising a sequence encoding an insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin.

The B(1-29)-A(1-21) encoding sequence may be provided by deletion of an internal sequence of the proinsulin gene (corresponding to amino acids 30 to 65) by site specific mutagenesis.

According to a second aspect of the present invention there is provided a replicable expression vehicle capable of expression of a DNA-sequence comprising a sequence encoding the insulin precursor containing the above peptide chain B(1-29)-A(1-21) of human insulin.

The expression vehicle may be a plasmid capable of replication in the host microorganism or capable of integration into the host organism chromosome. The vehicle employed may code for expression of repeated sequences of the desired DNA-sequence, each separated by selective cleavage sites.

According to a third aspect of the present invention there is provided a process for producing an insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin wherein a transformant microorganism including at least one expression vehicle capable of expressing the above insulin precursor is cultured in a suitable nutrient medium followed by isolation of the insulin precursor.

According to a fourth aspect of the present invention there is provided a microorganism transformed with an expression vehicle capable of expressing a DNA-sequence comprising a sequence encoding the above insulin precursor in the transformant microorganism.

By incorporation of the above expression vehicle into yeast considerable amounts of the present insulin precursor are produced. However, also prokaryotes such as Bacillus and E. coli strains are contemplated as host microorganisms.

The insulin precursor may be expressed with additional protein proceeding the insulin precursor. The additional protein may have the function of protecting the insulin precursor against, e.g. in vivo degradation by endogeneous enzymes or of providing information necessary to transport the desired protein into the periplasmic space and finally across the cell wall into the medium.

The additional protein contains a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor enabling subsequent splitting off the additional protein either by the microorganism itself or by later enzymatical or chemical cleavage.

Accordingly the present invention includes a DNA-sequence encoding the above insulin precursor and further comprising an additional DNA-sequence positioned upstream to the sequence encoding the insulin precursor and encoding an additional amino acid-sequence containing a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor.

According to a preferred embodiment of the present invention the additional amino acid sequence comprises at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor.

When the insulin precursor is expressed in yeast the additional amino acid-sequence may contain two basic amino acids (e.g. Lys-Lys, Arg-Arg, Lys-Arg or Arg-Lys) adjacent to N-terminal of the B'-chain of the insulin precursor, yeast being able to cleave the peptide bond between the basic amino acids and the precursor. Also a Glu-Ala or Asp-Ala cleavage site adjacent to the desired protein enables separation of the additional amino acid-sequence by the yeast itself by means of a dipeptidase enzyme produced by the yeast.

The insulin precursor may be secreted with an amino acid-sequence linked to the B(1-29)-chain of the precursor provided that this amino acid sequence contains a selective cleavage site adjacent to the B(1-29)-chain for later splitting of the superfluous amino acid sequence. As the insulin precursor does not contain methionine cyanogen bromide cleavage at

methionine adjacent to the desired protein would be operative. Likewise, arginine- and lysine-cleavage sites adjacent to the desired protein enables cleavage with trypsinlike proteases.

For secretion purposes the DNA-sequence encoding the insulin precursor may be fused to an additional DNA-sequence coding for a signal peptide. The signal peptide is cleaved off by the transformant microorganism during the secretion of the expressed protein product from the cells ensuring a more simple isolation of the desired product.The secreted product may be the insulin precursor or may contain an additional N-terminal amino acid-sequence to be removed later as explained above.

Secretion may be provided by including in the expression vehicle the yeast MFα1 leader sequence (Kurjan, J. and Herskowitz, I., Cell 30, (1982), 933 - 943) and according to a further preferred embodiment of the present invention the additional amino acid-sequence positioned upstream to the sequence encoding the insulin precursor comprises the yeast MFα1 leader coding sequence or part thereof.

The expression of the desired DNA-sequence will be under control of a promoter sequence correctly positioned to the DNA-sequence encoding the desired protein product to result in expression of the desired protein in the host organism. Preferably a promoter from a gene indigeneous to the host organism may be used. The DNA-sequence for the desired protein will be followed by a transcription terminator sequence, preferably a terminator sequence from a gene indigeneous to the host organism. If yeast is used as host organism the promoter and terminator sequences are preferably the promoter and terminator of the triose phosphase isomerase (TPI) gene, respectively.

Other promoters may be utilized such as the phosphoglycerate kinase (PGK1)- and the MFα1-promoter.

The insulin precursor according to the present invention may be converted into mature human insulin by transpeptidation with an L-threonine ester in the presence of trypsin or a trypsin derivative as described in the specification of Danish patent application 574/80 (the disclosure of which is incorporat-

ed by reference hereinto) followed by transformation of the threonine ester of human insulin into human insulin by known processes.

If the insulin precursor is secreted with an additional amino acid sequence adjacent to the N-terminal of the B(1-29)-chain such amino acid sequence should either be removed in vitro before the transpeptidation or should contain at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain as trypsin will cleave the peptide bond between the basic amino acid and the amino group of Phe^{B1} during the transpeptidation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate a preferred embodiment of the present invention.

Fig. 1 illustrates the preparation of plasmid pMT344, fig. 2 illustrates the preparation of plasmid pMT475, fig. 3 illustrates the preparation of plasmid pMT212, fig. 4 illustrates the preparation of plasmid pMT479 and fig. 5 illustrates the preparation of plasmid pMT319.

In the drawings and part of the following description the expression B' is used in stead of B(1-29) and A in stead of A(1-21). Accordingly the expression B'A is equivalent to the expression B(1-29)-A(1-21).

DETAILED DESCRIPTION

1. Preparation of a gene coding for human insulin

Total RNA purified (Chirgwin, J.M. Przybyla, A.E., McDonald, R.J. & Rutter, W.J., Biochemistry 18, (1979) 5294 - 5299) from human pancreas was reverse transcribed (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J.F. & Marcker, K.A., Proc.Natl.Acad.Sci. USA 80, (1983), 2866 - 2869) with AMV reverse transcriptase and d(GCTTTATTCCATCTCTC) as 1. strand primer. After preparative urea-polyacrylamide gel purification of the human insulin cDNA, the second strand was

synthesized on this template with DNA polymerase large fragment and d(CAGATCACTGTCC) as primer. After S1 nuclease digestion the human insulin ds. cDNA was purified by polyacrylamide gel electrophoresis, tailed with terminal transferase and cloned in the PstI site on pBR327 (Sorberon et al., Gene 9, (1980), 287 - 305) in E. coli. A correct clone harbouring the plasmid was identified from the recombinants by restriction endonuclease analysis and confirmed by nucleotide sequencing (Maxam, A., & Gilbert, W., Methods in Enzymology, 65 (1980), 499 - 560. Sanger, F., Nicklen, S. & Coulson, A.R., Proc.Natl.Acad.Sci. USA 74, (1977), 5463 - 5467).

2. Preparation of a gene coding for B(1-29)-A(1-21) of human insulin.

The gene encoding B(1-29)-A(1-21) of human insulin was made by site specific mutagenesis of the human insulin sequence with a 75bp in frame deletion in the C-peptide coding region inserted into a circular single stranded M-13 bacteriophage vector. A modified procedure (K. Norris et al., Nucl.Acids.Res. 11 (1983) 5103 - 5112) was used in which a chemically synthesized 19-mer deletion primer was annealed to the M13 template. After a short enzymatic extension reaction a "universal" 15-mer M13 dideoxy sequencing primer was added followed by enzymatic extension and ligation. A double stranded restriction fragment (BamHl-Hind III) was cut out of the partly single stranded circular DNA and ligated into pBR322 cut with BamHI and Hind III.

The obtained ligation mixture was used to transform E. coli and transformants harbouring a plasmid pMT319 containing the gene encoding B(1-29)-A(1-21) of human insulin was identified.

3. Plasmid constructions.

The gene encoding B(1-29)-A(1-21) of human insulin (B'A) was isolated as a restriction fragment from pMT319 and combined with fragments coding for the TPI promoter (T. Alber and

G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434), the MFα1 leader sequence (J. Kurjan and I. Herskowitz,. Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) and the transcription termination sequence from TPI of S.cerevisiae. These fragments provide sequences to ensure a high rate of transcription for the B'A encoding gene and also provide a presequence which can effect the localization of B'A into the secretory pathway and its eventual excretion into the growth medium. This expression unit for B'A ($TPI_{promoter}$-MFα1 leader - B'A - $TPI_{terminator}$) was then placed on a plasmid vector containing the yeast 2μ origin of replication and a selectable marker, LEU 2, to give pMT344, a yeast expression vector for B'A.

During in vivo maturation of α-factor in yeast, the last (C-terminal) six amino acids of the MFα1 leader peptide (Lys-Arg-Glu-Ala-Glu-Ala) are removed from the α-factor precursor by the sequential action of an endopeptidase recognizing the Lys-Arg sequence and an aminodipeptidase which removes the Glu-Ala residues (Julius, D. et al. Cell 32 (1983) 839 - 852). To eliminate the need for the yeast aminodipeptidase, the sequence coding for the C-terminal Glu-Ala-Glu-Ala of the MFα1 leader was removed from pMT344 via in vitro mutagenesis. The resulting yeast expression plasmid, pMT475, contains the insert coding for $TPI_{promotor}$-MFα1 leader (minus Glu-Ala-Glu-Ala) - B'A - $TPI_{terminator}$.

In a preferred construction the modified expression unit was transferred to a stable, high copy number yeast plasmid CPOT, which can be selected merely by the presence of glucose in the growth medium. The resulting yeast expression vector for B'A was numbered pMT479.

4. Transformation

Plasmids pMT344 and pMT475 were transformed into S. cerevisiae leu 2 mutants by selection for leucin prototrophy as described by Hinnen et al.(A. Hinnen, J.B. Hicks and G.R. Fink. Transformation of Yeast. Proc.Nat.Aca.Sci. 75 (1978) 1929).

Plasmid pMT479 was transformed into S. cerevisiae strains carrying deletions in the TPI gene by selecting for growth on glucose. Such strains are normally unable to grow on glucose as the sole carbon source and grows very slowly on galactose lactate medium. This defect is due to a mutation in the triose phosphate isomerase gene, obtained by deletion and replacement of a major part of this gene with the S. cerevisiae LEU 2 gene. Because of the growth deficiencies there is a strong selection for a plasmid which contains a gene coding for TPI. pMT479 contains the Schizo. pombe TPI gene.

5. Expression of B(1-29)-A(1-21) insulin in yeast

Expression products of B(1-29)-A(1-21) insulin type were measured by radioimmunoassay for insulin as described by Heding, L. (Diabetologia 8, 260 - 66, 1972) with the only exception that a B(1-29)-A(1-21) insulin standard was used instead of an insulin standard. The B(1-29)-A(1-21) insulin standard was purified from yeast supernatant from strain MT 519 (pMT 479) (see Example 9). The purity of the standard was 98% as determined by HPLC and the actual concentration of peptide in the standard was determined by amino acid analysis (see Example 9). The expression levels of immunoreactive B(1-29)-A(1-21) insulin in the transformed yeast strains are summarized in Table 1.

Table 1

Expression levels of immunoreactive B(1-29)-A(1-21) insulin in yeast

Yeast strain	Plasmid	Immunoreactive B(1-29)-A(1-21) insulin (nmol/l supernatant)
MT 350 (DSM 2957)	pMT 344	100
MT 371 (DSM 2958)	pMT 475	192
MT 519 (DSM 2959)	pMT 479	2900

The isolation and characterization of B(1-29)-A(1-21) insulin type of expression products are given in Examples 7 - 9.

6. Conversion of B(1-29)-A(1-21) insulin into B30 esters of human insulin

The conversion of B(1-29)-A(1-21) insulin into human insulin esters can be followed quantitatively by HPLC (high pressure liquid chromatography) on reverse phase. A 4 x 300 mm "μBondapak C18 column" (Waters Ass.) was used and the elution was performed with a buffer comprising 0.2 M ammonium sulphate (adjusted to a pH value of 3.5 with sulphuric acid) and containing 26 - 50% acetonitrile. The optimal acetonitrile concentration depends on which ester one desires to separate from B(1-29)-A(1-21) insulin.

In case of human insulin methyl ester separation is achieved in about 26% (v/v) of acetonitrile. B(1-29)-A(1-21) insulin and Thr-OMe^{B30} human insulins elute after 2 and 5.9 column volumes, respectively, as well separated symmetrical peaks.

Before the application on the HPLC column the proteins in the reaction mixture were precipitated by addition of 10 volumes of acetone. The precipitate was isolated by centrifugation, dried in vacuo, and dissolved in 1 M acetic acid.

EXPERIMENTAL PART

Example 1

Construction of a gene coding for B(1-29)-A(1-21)insulin

Materials and Methods

15-mer M13 dideoxy sequencing primer d(TCCCAGTCACGACGT), T4 DNA ligase and restriction enzymes were obtained from New England Biolabs. DNA polymerase I "Klenow fragment" and T_4 polynucleotide kinase were purchased from P-L Biochemicals. (γ-^{32}P)-ATP (7500 Ci/mmol) was obtained from New England Nuclear. The support for oligonucleotide synthesis was 5'-O-dimethoxytrityl N^2-isobutyryldeoxyguanosine bound via a 3'-O-succinyl group to aminomethylated 1% crosslinked polystyrene beads from Bachem.

Construction of M13 mp10 insHX PstΔphage:

The M13 mp10 derived phage mp10 insHX was constructed by cloning of the 284 bp large proinsulin coding Hind III-XbaI fragment, isolated from p285, into Hind III-XbaI cut M13 mp10 RF (Messing, J. and Vieira, J. (1983) Unpublished results).

M13 mp10 insHXΔPst was constructed from mp10 insHX,RF by complete PstI digestion followed by ligation and transformation of *E. coli* JM103. The resulting phage harbours the human proinsulin coding sequences, with a 75 bp in frame deletion in the C-peptide coding region. Single stranded phage was prepared as described (Messing, J. and Vieira, J. (1982) Gene 19, 269 - 276).

Oligodeoxyribonucleotide synthesis

The 19-mer deletion primer d(CACACCCAAGGGCATTGTG) was synthesized by the triester method on a 1% crosslinked polystyrene support (Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) Nucl.Acids Res. 10, 1755 - 1769). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an HPLC pump and a control module. The oligonucleotide was purified after deprotection by HPLC on a

LiChrosorb RP18 column (Chrompack (Fritz, H.-J., Belagaje, R., Brown, E.L., Fritz, R.H., Jones, R.A., Lees, R.G., and Khorana, H.G. (1978) Biochemistry 17, 1257 - 1267).

5'-^{32}P-labelling of oligodeoxyribonucleotide

The 19-mer was labelled at the 5'end in a 60µl reaction mixture containing 50 mM Tris-HCl at pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 0.4% glycerol, 120 pmole ATP, 50 µCi of (γ-^{32}P)-ATP (10 pmole), 120 pmole of oligonucleotide and 30 units of T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min., and terminated by heating at 100°C for 3 min. The labelled oligonucleotide was separated from unreacted (γ-^{32}P)-ATP by chromatography on a column (1 x 8 cm) of Sephadex G50 superfine in 0.05 M triethylammonium bicarbonate at pH 7.5.

For colony hybridization the oligonucleotide was labelled without the addition of "cold" ATP as described (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J., and Marcker, K. (1983) Proc.Natl.Acad.Sci. USA 80, 2866 - 2869).

Oligodeoxyribonucleotide primed DNA synthesis

Single stranded M13 mp10 insHXΔPst (0.4 pmole was incubated with the 19-mer 5'-(^{32}P)-labelled oligodeoxyribonucleotide primer (10 pmole) in 20 µl of 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DDT for 5 min. at 55°C and annealed for 30 min. at 11°C. Then 9 µl of d-NTP-mix consisting of 2.2 mM of each dATP, dCTP, dGTP, dTTP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DDT was added followed by 7 units of E. coli DNA polymerase I (Klenow). The mixture was kept for 30 min. at 11°C and heated for 10 min. at 65°C. 15-mer universal primer for dideoxy sequencing (4 pmole) was added and the mixture heated at 65°C for an additional minute. After cooling to 11°C 26 µl of solution containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.8 mM of each dATP, dCTP, dGTP, dTTP, 2.4 mM ATP and 10^3 units of T4 ligase was added followed by 9.5 units of E. coli DNA polymerase I (Klenow). The final volume of the mixture was 64 µl.

After incubation for 3 hours at 11°C 20 µl 4M sodium acetate was added, and the volume adjusted to 200 µl with TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The mixture was extracted twice with phenol/chloroform. 0.9 µg (0.3 pmole) of the purified large fragment of pBR322 cleaved with BamHI and Hind III was added as carrier DNA. After ether extraction of the aqueous phase, the DNA was isolated by ethanol precipitation.

Endonuclease digestion

The DNA, prepared as described above, was digested respectively with 16 and 20 units of restriction endonucleases BamHI and Hind III in a total volume of 22µl of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DDT, 4 mM spermidine). The mixture was extracted with phenol/chloroform followed by ether and the DNA was isolated by ethanol precipitation and then dissolved in 12 µl H_2O. 2µl was used for electrophoresis on a 7M urea 6% polyacrylamide gel.

Ligation

To a part of the DNA (5 µl) was added a new portion of the purified large fragment of pBR322 cut with BamHI and Hind III (0.38 µg) and 400 units of T4 DNA ligase, in a total volume of 41 µl containing 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DDT, 40 µg/ml gelatine. Ligation was performed at 16°C for 16 hours.

Transformation

20.5 µl of the ligation mixture was used to transform $CaCl_2$ treated E. coli MC 1000 (r^-, m^+). The bacteria were plated on LB-agar plates and selected for resistance to ampicillin (100 µg/ml). 2.6×10^3 colonies per pmole of M13 mp10 insHX Pst were obtained.

Colony hybridisation

123 transformed colonies were picked onto fresh ampicillin plates and grown overnight at 37°C. Colonies were transferred to Whatman 540 filter paper and fixed (Gergen, J.P., Stern, R.H., and Wensink, P.C. (1979), Nucl.Acids Res. 7, 2115 - 2136). A prehybridization was performed in a sealed plastic bag with 6 ml of 0.9 M NaCl, 0.09 M Tris-HCl pH 7.5 0.006 M EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1% SDS and 50 µg/ml salmon sperm DNA for 2 hours at 65°C. Then 8.5 x 10^6 cpm of ^{32}P-labelled 19-mer was added and hybridisation performed at 45°C overnight. The filter was washed with 0.9 M NaCl, 0.09 M sodium citrate three times at 0°C for 5 min. and was then autoradiographed and washed once at 45°C for 1 min. and autoradiographed again. After washing at 45°C, identification of 3 colonies containing mutated plasmid was possible.

Endonuclease analysis of mutated plasmids

Plasmids from the supposed mutant colonies were prepared by a rapid method (Ish-Horowicz, D. and Burke, J.F. (1981), Nucl.Acids Res. 9, 2989 - 2998), digested with a mixture of BamHI and Hind III and then analysed by electrophoresis on a 2% agarose gel. The presence of a 179 bp fragment confirmed that the 3 colonies contained mutant plasmid.

Retransformation

The colonies identified as "mutant" contain plasmids which are the progeny of a heteroduplex. Pure mutant could be obtained by retransformation of $CaCl_2$ treated *E. coli* MC1000 (r^-, m^+)with plasmid from 2 of the mutant colonies. From each plate 5 ampicillin resistant clones were isolated, plasmid DNA was prepared and analysed by endonuclease cleavage as mentioned above. 3 out of 5 and 5 out of 5 respectively were shown to be pure mutant. One plasmid pMT319 was selected for further use.

DNA sequence analysis

5 µg of pMT319 was cleaved with BamHI under standard conditions, phenol extracted and ethanol precipitated. Filling in of the BamHI sticky ends was performed with Klenow DNA polymerase I, dCTP, dGTP, dTTP, and α-^{32}P-dATP.

After phenol extraction and ethanol precipitation the DNA was digested with EcoRI. The ^{32}-P labelled fragment with the deletion was purified by electrophoresis on a 2% agarose gel and sequenced by the Maxam-Gilbert method (Maxam, A. and Gilbert, W. (1980) Methods in Enzymology 65, 499 - 560).

Example 2

Construction of a yeast plasmid pMT344 for expression of B(1-29)-A(1-21) of human insulin (B'A).

Plasmid pMT319 containing the gene coding for B'A and constructed as explained above was cut with restriction enzymes Hind III and XbaI and a 0.18 kb fragment was isolated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) from a 2% agarose gel. Similarly a fragment (6.5 kb XhoI - Hind III) containing the S. cerevisiae TPI promotor (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434) and the MFα1 leader sequence (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) was isolated from plasmid p285 constructed as described in US-patent application S.N. 547,748 of November 1, 1983. A fragment (0.7 kb XbaI - BamHI) containing the TPI transcription termination sequences (T. Alber and G. Kawasaki, Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434) was also isolated from p285. Finally a 5.4 kb XhoI - BamHI fragment was isolated from the yeast vector YEp13 (J.R. Broach. Construction of High Copy Yeast Vectors Using 2µm Circle Sequences. Methods Enzymology 101 (1983) 307 - 325). The above four fragments were ligated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold

Spring Harbor Press 1982) and transformed into E. coli (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) selecting for ampicillin resistance. Plasmids were isolated from the transformants and the structure of one of these, pMT344, verified by restriction mapping. The construction and main features of pMT344 are outlined in fig. 1.

Example 3

Construction of a yeast plasmid pMT475 for expression of B(1-29)-A(1-21) of human insulin (B'A) after a modified MFα1 leader.

To construct a plasmid for the expression of B'A after a MFα1 leader (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) lacking its last four amino acids (Glu Ala Glu Ala), the 0.14 kb XbaI - EcoRII fragment containing the A and part of the B' sequences was isolated from pMT319. Likewise the 5' proximal part of the B' gene was isolated as a 0.36 kb EcoRI - EcoRII fragment from pM215. This plasmid was constructed by subcloning the EcoRI - XbaI fragment containing the insulin BCA gene from p285 into pUC13 (constructed as described for pUC8 and pUC9 by Vieira et al., Gene 19: 259 - 268 (1982)) and subsequent in vitro loop-out removal of the 12 bases coding for Glu Ala Glu Ala at the junction between MFα1 leader and insulin BCA. These two pieces covering the B'A gene were ligated to EcoRI - XbaI digested pUC13 vector (see fig. 2) to give pMT473. The modified gene contained within a 0.5 kb EcoRI - XbaI fragment was isolated from pMT473 and then ligated to two fragments (4.3 kb XbaI - EcoRV and 3.3 kb EcoRV - EcoRI) from pMT342. pMT342 is the yeast vector pMT212 with an inserted $TPI_{promotor}$-MFα1 leader - BCA - $TPI_{terminator}$. The resulting plasmid, pMT475, contains the insert: $TPI_{promotor}$ - MFα1 leader (÷ Glu Ala Glu Ala) - B'A - $TPI_{terminator}$. The construction of plasmids pMT342, 473 and 475 is outlined in fig. 2. The construction of the vector pMT212 is shown in fig. 3. Plasmid pMLB1034 is described by M.L. Berman et al., Advanced Bacterial Genetics, Cold Spring Harbor (1982), 49 - 51.

Example 4

Insertion of the B(1-29)-A(1-21) (B'A) gene into a stable yeast plasmid pMT479.

The modified B'A gene from pMT475 was isolated as a 2.1 kb BamHI - partial Sphl fragment and ligated to an approximately 11 kb BamHl - Sphl fragment of plasmid CPOT to give plasmid pMT479 (fig. 4). Plasmid CPOT is based on the vector Cl/1 which has been modified by substituting the original pBR322 Bgll - BamHI fragment with the similar Bgll - BamHI fragment from pUC13 and subsequent insertion of the S.pombe TPI gene (US patent application S.N. filed on 25th May,1984) as a BamHI - SalI fragment to give CPOT. Cl/1 is derived from pJDB 248, Beggs et al., Nature 275, 104 - 109 (1978) as described in US patent application S.N. 489,406, filed April 28, 1983).

Example 5

Transformation

S. cerevisae strain MT118 (a, leu 2, ura 3, trp 1) was grown on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) to an $O.D._{600}$ of 2.1. 100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of (1.2 M sorbitol, 25 mM Na_2EDTA pH= 8.0, 6.7 mg/ml dithiotreitol). The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of (1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate pH = 5.8, 2 mg Novozym® 234). The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and in 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (Tris = Tris(hydroxymethyl)-aminometan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of plasmid pMT344 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v

YPD, 6.7 mM $CaCl_2$, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) with leucine omitted and containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant MT350 (=MT 118/pMT344) was chosen for further characterization.

Plasmid pMT475 was transformed into S.cerevisiae strain MT 362 (α,leu2) by the same procedure as above, and the transformant MT371 (=MT362/pMT475) isolated.

Transformation of pMT479 into strain E2-7B X E11-3C (a/α, Δtpi/Δtpi, pep 4-3/pep 4-3; this strain will be referred to as MT501) was performed as above with the following modifications: 1) prior to transformation strain MT501 was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an $O.D._{600}$ of 0.6. 2) the SOS solution contained YPGaL instead of YPD. One transformant MT519 (=MT501/pMT479) was chosen for further characterization.

The transformed microorganisms MT 350, MT 371 and MT 519 were deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), Griesebachstrasse 8, D-3400 Göttingen, on May 15, 1984 and accorded the reference numbers DSM 2957, DSM 2958, and DSM 2959, respectively.

Example 6

Expression of B(1-29)-A(1-21) insulin in yeast

Strains MT350 (DSM 2957) and MT371 (DSM 2958) were grown in synthetic complete medium SC (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981) with leucine omitted. For each strain, two 1 liter cultures in 2 liter baffled flasks were shaken at 30°C until they reached $O.D._{600nm}$ of 7 to 10. They were then centrifuged and the supernatant removed for further analysis.

Strain MT519 (DSM 2959) was grown similarly but on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) and to an $O.D._{600nm}$ of 15, centrifuged and the supernatant separated for analysis as above.

Example 7

Expression of B(1-29)-A(1-21) insulin in yeast strain MT350 (DSM 2957)

Yeast strain MT350 (DSM 2957) was grown as previously described in example 6 and expression products from 1100 ml of supernatant from this strain were isolated as follows:

10 g of LiChroprep® RP-18 (Merck, art. 9303) were washed 3 times with 50 mM NH_4HCO_3, 60% EtOH and thereafter packed in a 6 x 1 cm column. The column was equilibrated with 50 ml of 50 mM NH_4HCO_3. 55 ml of 96% EtOH were added to 1100 ml of the yeast supernatant, and the mixture was applied to the column overnight (flow: 70 ml/h).

The column was washed with 10 ml of 0.5 M NaCl and 10 ml of H_2O, and the peptides were eluted with 50 mM of NH_4HCO_3, 60% EtOH. The eluate (5 ml) was concentrated by vacuum centrifugation to 1.4 ml (to remove the ethanol), and the volume was adjusted to 10 ml with 25 mM of HEPES buffer pH = 7.4. The sample was applied to an antiinsulin immunoabsorption column (AIS column) (2.5 x 4.5 cm) which had been washed 4 times with 5 ml of NaFAM-buffer (Heding, L., Diabetologia 8, 260-66, 1972) and twice with 5 ml of 25 mM HEPES-buffer prior to the application. After the application, the column was allowed to stand for 30 min. at room temperature and was thereafter washed 10 times with 4 ml of 25 mM HEPES buffer. The peptides were eluted with 20% HAc. The pH value of the eluate was adjusted to 7.0 with NH_4OH, and the pool was concentrated to 500 µl by vacuum rotation.

The sample from the previous step was further purified on HPLC on a 10µ Waters µBondopak C-18 column (3.9 x 300 mm). The A and B buffers were 0.1% TFA in H_2O and 0.07% TFA in MeCN, respectively. The column was equilibrated with 25% B (flow: 1.5 ml/min.) and the peptides were eluted with a linear gradient of MeCN (1%/min.) and detected at 276 nm. The yield in each step of

the purification was determined by radioimmunoassay as previously described, and Table 2 summarizes the purification. The overall yield was 68%.

Table 2

Purification of expression products from yeast strain MT350 supernatant

Purification step	Volume (ml)	Immunoreactive B(1-29)-A(1-21) insulin (nmol)
Supernatant	1100	110[x]
RP-18	10	116
Anti-insulin Sepharose	0.5	116
HPLC	2.5	75

x) Dilution effect was observed in this sample

Only one peak containing immunoreactive B(1-29)-A(1-21) insulin material was detected from the HPLC column. Peptide material from this peak was isolated and subjected to amino acid sequence analysis. The sequence analysis was performed with a Gas Phase sequencer (Applied Biosystem Model 470A) as described by Hewick, R.M. et al. (J.Biol.Chem. 256, 7990-7997, 1981). From the sequencing results it could be concluded that the expression products consisted of 3 peptides:

$(Glu\text{-}Ala)_2$-B(1-29)-A(1-21) insulin	89%
Glu-Ala-B(1-29)-A(1-21) insulin	2%
B(1-29)-A(1-21) insulin	9%

The peptides were present in the relative amount as indicated.

Example 8

Expression of B(1-29)-A(1-21) insulin in yeast strain MT371 (DSM 2958)

Yeast strain MT371 (DSM 2958) was grown as previously described in example 6 and expression products from 665 ml of supernatant from this strain were isolated as described in Example 7. The overall yield was 50 nmol, corresponding to 39%. Peptide material was isolated from the HPLC column and sequenced as described in Example 7. From the sequence results (18 residues from the N-terminal) it could be concluded that the peptide was homogeneous B(1-29)-A(1-21) insulin.

Example 9

Expression of B(1-29)-A(1-21) insulin in yeast strain MT519 (DSM 2959)

Yeast strain MT519 (DSM 2959) was grown as previously described in example 6 and expression products from 70 ml of supernatant were isolated as described in example 7. The overall yield was 116 nmol, corresponding to 57%. The peptide was sequenced as described in Example 7. As judged from the 42 residues identified from the N-terminal end, the peptide was homogeneous B(1-29)-A(1-21) insulin. Approximately 5 nmol of peptide was hydrolyzed in 100 µl 6N HCl for 24 h at 110°C. The hydrolysate was analyzed on a Beckman Model 121M amino acid analyser. The following amino acid composition was found:

Table 3

Amino acid analysis of purified B(1-29)-A(1-21) insulin

Amino acid	Found	Theory	Amino acid	Found	Theory
Asx*	2.97	3	Val	3.37	4
Thr	1.77	2	Ile	1.65	2
Ser	2.45	3	Leu*	5.65	6
Glx*	6.68	7	Tyr	3.51	4
Pro	1.33	1	Phe*	2.73	3
Gly*	3.95	4	Lys*	0.95	1
Ala*	1.22	1	His*	1.84	2
Cys 0.5	4.54	6	Arg*	1.13	1

*) amino acid used for normalization.

Example 10

20 mg of B(1-29)-A(1-21) insulin (from MT519 (DSM 2959)) was dissolved in 0.1 ml of 10 M acetic acid and 0.26 ml of 1.54 M Thr-OMe in N,N-dimethylacetamide was added. The mixture was cooled to 12°C. 2 mg of trypsin dissolved in 0.035 ml of 0.05 M calcium acetate was added. After 6 days at 12°C the proteins were precipitated by addition of 5 ml of acetone. The conversion of B(1-29)-A(1-21) insulin into Thr-OMe^{B30} human insulin was 15% as adjusted by HPLC.

CLAIMS

1. A DNA-sequence comprising a sequence encoding an insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin.

2. A DNA-sequence according to claims 1 containing an additional DNA-sequence positioned upstream to the sequence encoding the insulin precursor and encoding an additional amino acid-sequence containing a selective cleavage site adjacent to the N-terminal of the B'-chain of the insulin precursor.

3. A DNA-sequence according to claim 2 wherein the additional amino acid sequence comprises at least one basic amino acid adjacent to the N-terminal of the B'-chain of the insulin precursor.

4. A DNA-sequence according to claim 2 wherein the additional amino acid sequence comprises Glu-Ala or Asp-Ala adjacent to the N-terminal of the B'-chain of the insulin precursor.

5. A DNA-sequence according to claim 2 wherein the additional amino acid sequence comprises methionine adjacent to the N-terminal of the B'-chain of the insulin precursor.

6. A DNA-sequence according to claim 2 wherein the additional amino acid sequence comprises a signal peptide sequence.

7. A DNA-sequence according to claim 6 wherein the signal peptide comprises the yeast MFα1 leader coding sequence or part thereof.

8. A replicable expression vehicle capable of expressing a DNA-sequence according to claims 1 to 7 in a transformant microorganism.

9. A replicable expression vehicle according to claim 8 being under the control of the yeast promoter TPI.

10. A replicable expression vehicle according to claims 8 - 9 comprising the plasmid pMT344 illustrated in Fig. 1 hereof.

11. A replicable expression vehicle according to claims 8 - 9 comprising the plasmid pMT475 illustrated in Fig. 2 hereof.

12. A replicable expression vehicle according to claims 8 - 9 comprising the plasmid pMT 479 illustrated in Fig. 4 hereof.

13. A microorganism transformed with an expression vehicle according to claims 8 - 12.

14. A process for producing an insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin, wherein a transformant microorganism including at least one replicable expression vehicle according to claims 8 - 12 is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

15. A process according to claim 14 wherein the transformant microorganism is yeast.

16. A process according to claim 14 wherein the transformant microorganism is Bacillus.

17. A process according to claim 14 wherein the transformant microorganism is E. coli.

18. A process for producing an insulin precursor according to claim 15 wherein a yeast strain DSM 2957 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

19. A process for producing an insulin precursor according to claim 15 wherein a yeast strain DSM 2958 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

20. A process for producing an insulin precursor according to claim 15 wherein a yeast strain DSM 2959 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

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A B S T R A C T

An insulin precursor containing the peptide chain B(1-29)-A(1-21) of human insulin is prepared by transforming a host microorganism with replicable expression vehicle capable of expressing a DNA-sequence encoding the insulin precursor. The preferred host microorganism is yeast.

FIG. 1

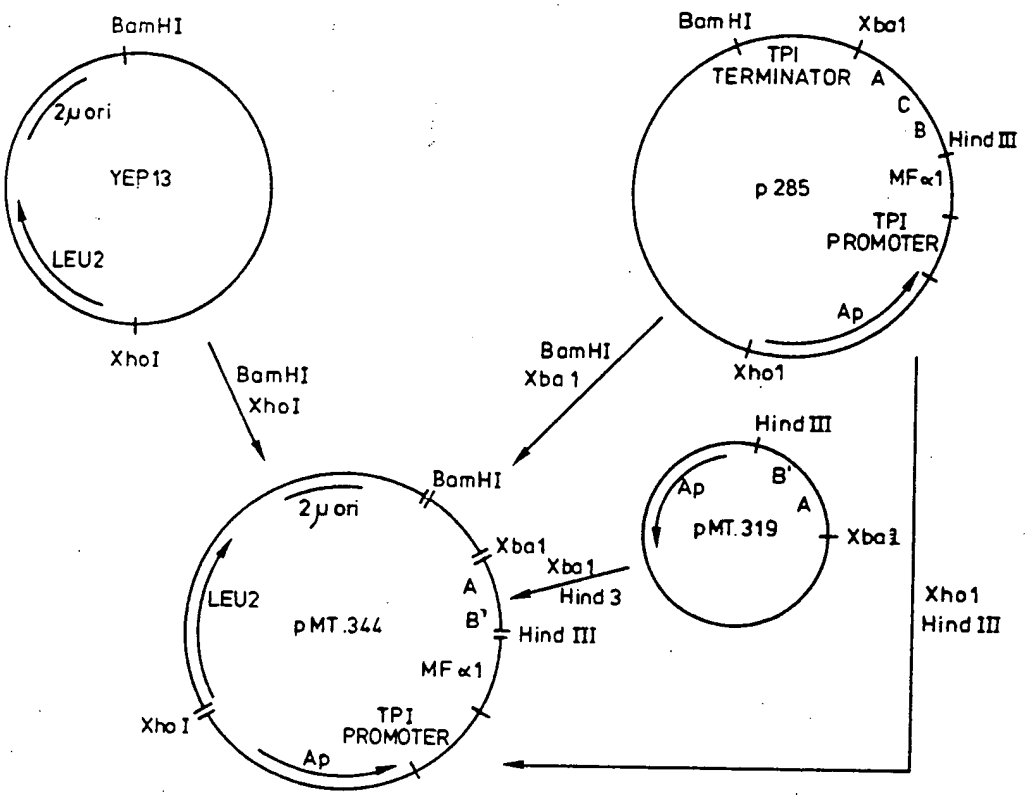
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FIG. 2

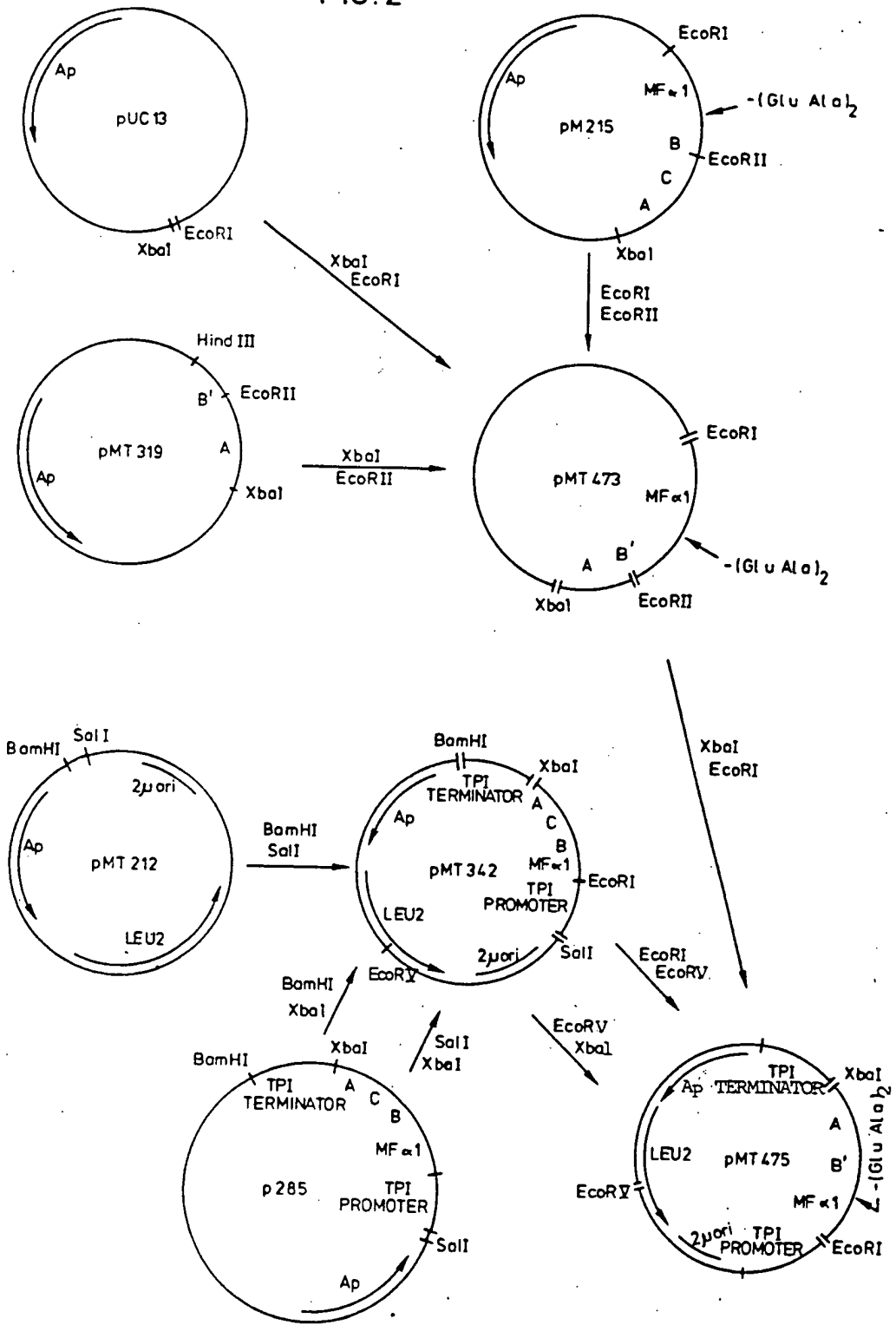
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FIG.3

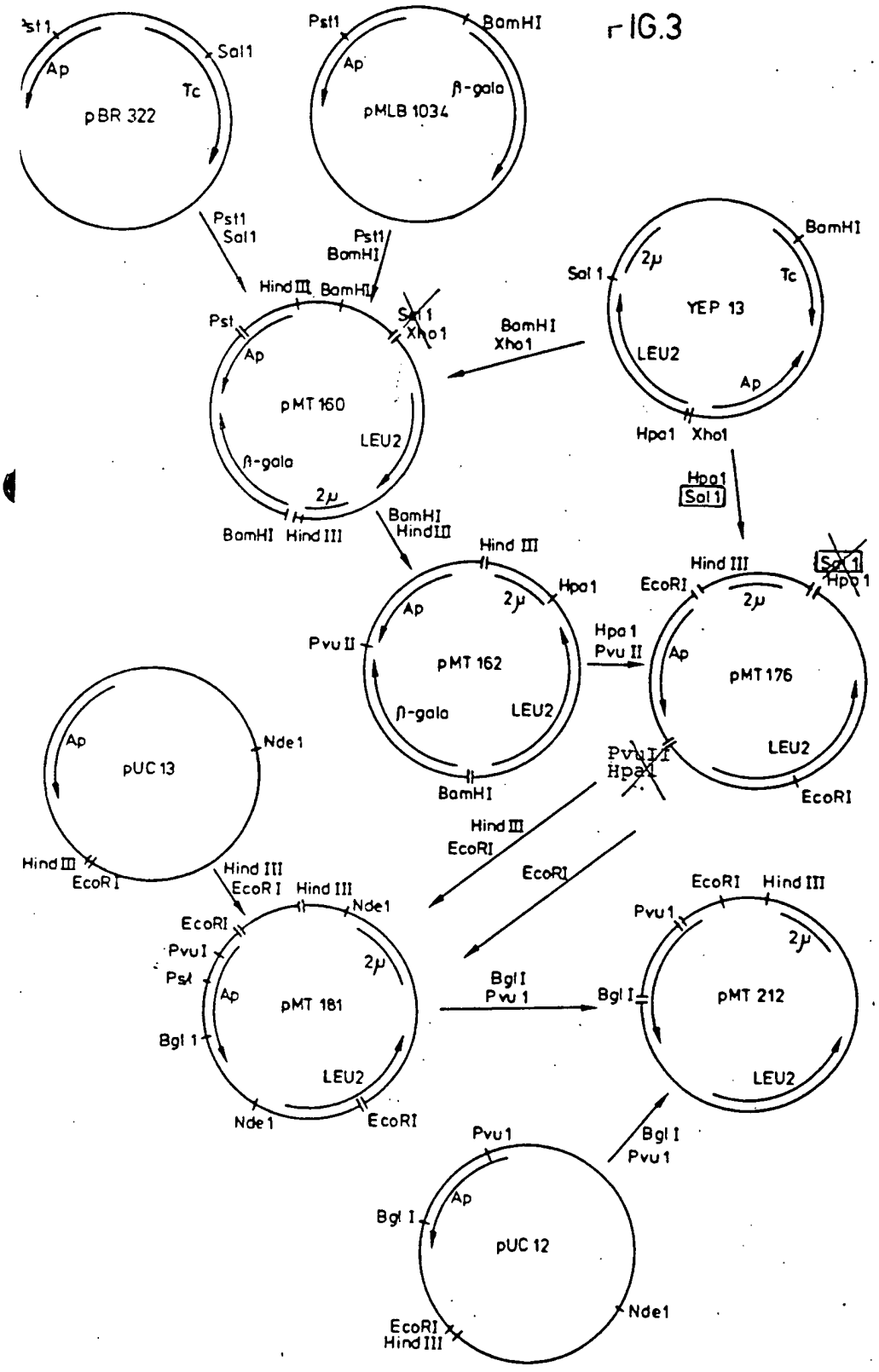
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FIG. 4

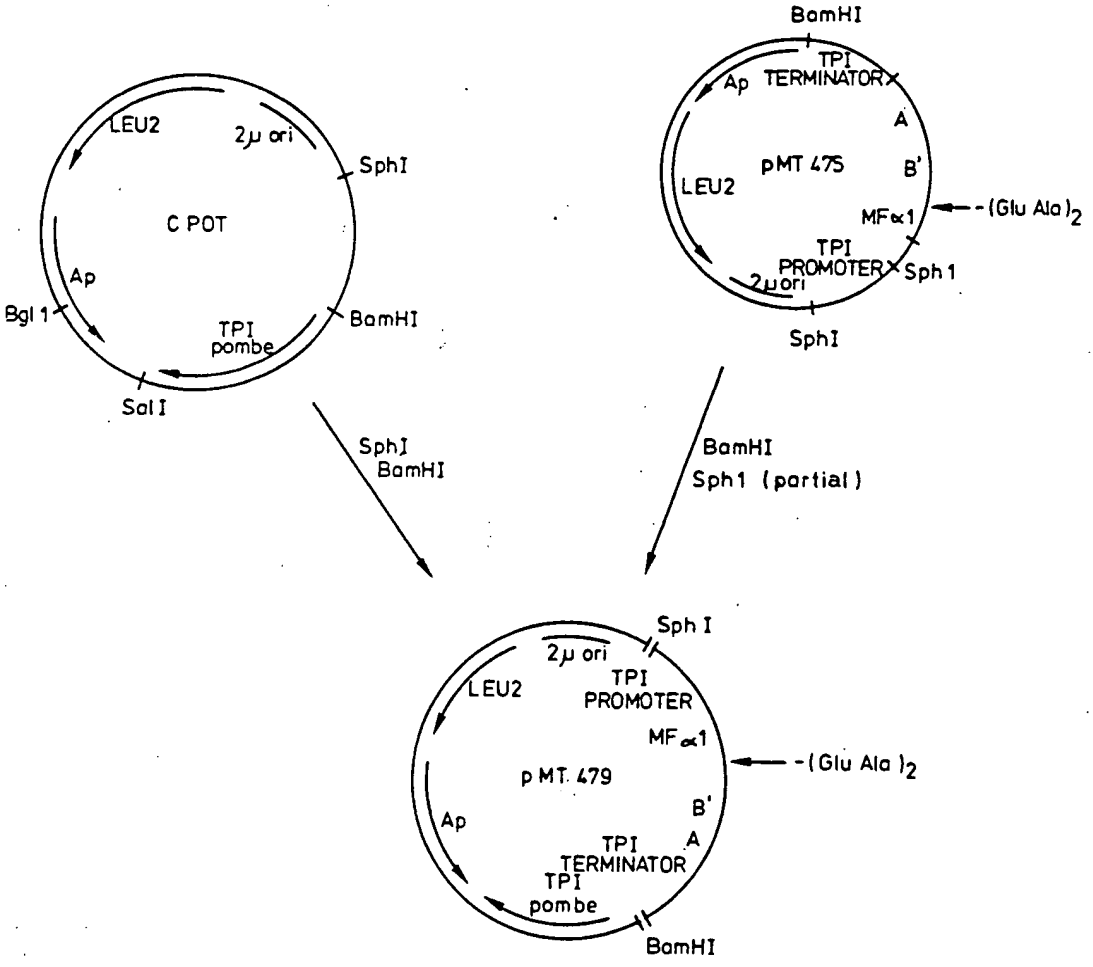
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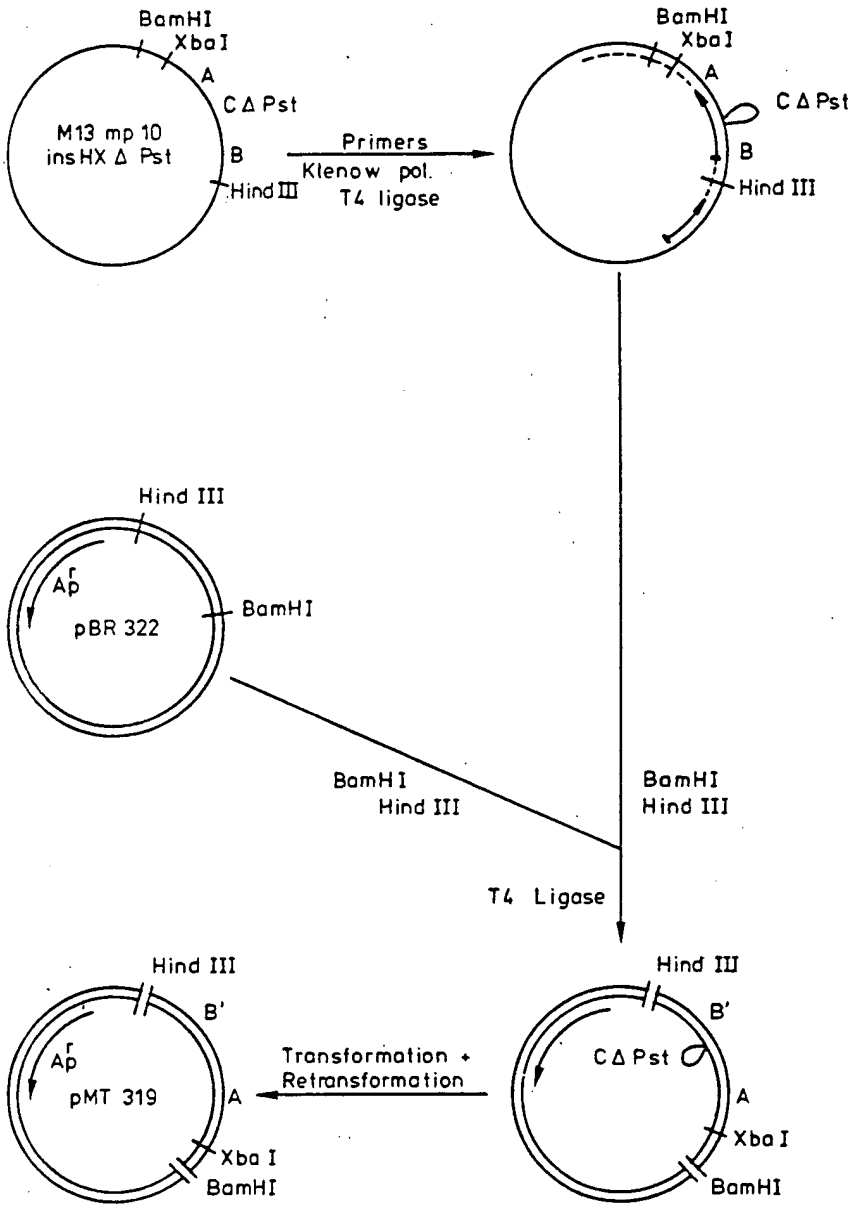
FIG. 5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KONGERIGET DANMARK

DIREKTORATET FOR PATENT- OG VAREMÆRKEVÆSENET

does hereby certify that on the 8th February, 1985, NOVO INDUSTRI A/S, Bagsværd, Denmark, has filed an application for a patent in Denmark for "DNA-sequence encoding biosyntetic insulin precusors and process for preparing the insulin precursors and human insulin".

(Application No. 582 of 1985. Int. Cl. C 12 N).

The attached photocopy is a true copy of the specification, abstract and 3 drawings filed with the application.

København, den 26th April, 1985

For the D.P.V.
by order

Minna Gram

Senior Clerk.

2620.010-DK
1985.01.22

ATS/KHaa
(ats 3)

5 DNA-sequence Encoding Biosynthetic Insulin Precursors and Process for Preparing the Insulin Precursors and human insulin.

This invention relates to biosynthetic insulin. More specifically, the invention is directed to a DNA-sequence encoding a biosynthetic insulin precursor and to the preparation of such insulin precursor which is convertible into biosynthetic insulin by a transpeptidation process.

BACKGROUND OF THE INVENTION

In the past insulin has been synthezised (from synthetic A- and B-chains) or re-synthesized (from naturally derived A- and B-chains) by combining the two chains in an oxidation process whereby the 6 cysteine sulfhydryl groups of the reduced chains (4 in the A-chain, 2 in the B-chain) are converted into disulfide bonds. By this method disulfide bonds are formed largely at random, meaning that the yield of insulin with disulfide bridges correctly positioned between cysteine residues A-6 and A-11, A-7 and B-7, and A-20 and B-19, respectively, is very low.

Following the discovery of proinsulin as a biological precursor of insulin it was observed that the A- and B-polypeptide moieties of the linear-chain totally reduced proinsulin (those moieties corresponding to the A- and B-chains of insulin, respectively) could be oxidatively combined with much less randomization of the disulfide bonds to give a substantially higher yield of correctly folded proinsulin as compared with the combination of free A- and B-chains (D.F. Steiner et al.: Proc.Nat.Acad.Sci. 60 (1968), 622). Albeit high yields were obtained only at proinsulin concentrations too low to make the process feasible on a preparative scale, the function of the C- (i.e. connecting peptide) moiety of the B-C-A polypeptide sequence of proinsulin, namely that of bringing the 6 cysteine residues into spatial positions favorable for correct oxidation into proinsulin, was clearly demonstrated.

The proinsulin formed may function as an *in vitro* precursor of insulin in that the connecting peptide is removable by enzymatic means (W. Kemmler *et al.*: J.Biol.Chem. 246 (1971), 6786).

Subsequently it has been shown that proinsulin-like compounds having shorter linking moieties than the C-peptide and flanked at both ends by specific enzymatic or chemical cleavage sites (the so-called miniproinsulins (A. Wollmer et al., Hoppe-Seyler's Z. Physiol.Chem. 355 (1974), 1471 - 1476 and Dietrich Brandenburg et al., Hoppe-Seyler's Z. Physiol.Chem. 354 (1973), 1521 - 1524)) may also serve as insulin precursors.

Endeavours to provide biosynthetic insulins, particularly that identical to the human species, have followed the same strategic pathways as those to synthetic insulin. The insulin A- and B-chains have been expressed in separate host organisms, isolated therefrom and then combined as described *supra* (R.E. Chance *et al.*: Diabetes Care 4 (1982), 147). Microorganisms have been transformed with cloning vectors encoding preproinsulin or proinsulin which may be secreted as such (W. Gilbert *et al.*: European Patent Publ. No. 6694) or accumulated intracellularly as hybrid gene products (D.V. Goeddel *et al.*: European Patent Publ. No. 55945). The miniproinsulin pathway has also been attempted (D.V. Goeddel, *supra*).

Procuring the A- and B-chains in separate fermentation processes followed by combination of the chains is inherently impractical. The dual fermentation inconvenience may be overcome by choosing the proinsulin or miniproinsulin strategy. However, the use of a proinsulin as the biosynthetic insulin precursor may entail certain disadvantages. The proinsulin, whether excreted into the fermentation liquid as such or accumulated intracellularly in the host organism, possibly as a hybrid gene product, is likely to contain substantially randomized disulfide bonds. The refolding of such "scrambled" products into correctly folded proinsulin may be conducted either directly (H.-G. Gattner *et al.*: Danish Patent Application No. 4523/83) or via the single chain hexa-S-sulfonate (F.B. Hill: European Patent Publ. No. 37255). The refolding

process usually entails some degree of polymerization and hence the inconvenience of using laborious purification steps during recovery.

In addition, insulin precursors of the proinsulin type are prone to undergo enzymatic degradation, either within the host cells or following its excretion into the fermentation broth. In yeast it has been shown that human proinsulin is particularly sensitive to enzymatic cleavages at the two dibasic sequences (Arg31-Arg32 and Lys64-Arg65). Apparently these cleavages occur before the establishment of the S-S bridges, resulting in the formation of C-peptide, A-chain and B-chain.

OBJECT OF THE INVENTION AND SUMMARY THEREOF

The object of the present invention is to circumvent these disadvantages by devising a biosynthetic insulin precursor which is generated largely with correctly positioned disulfide bridges between the A- and B-moieties and, furthermore, substantially more resistant to proteolytic degradation than the biosynthetic insulin precursors known heretofore.

A single chain insulin precursor consisting of a shortened insulin B-chain from Phe^{B1} to Lys^{B29} continuing into a complete A-chain from Gly^{A1} to Asn^{A21}, B(1-29)-A(1-21), is known (Jan Markussen, "Proteolytic degradation of proinsulin and of the intermediate forms",: Proceedings of the Symposium on Proinsulin, Insulin and C-Peptide, Tokushima, 12 - 14 July, 1978, Editors: S. Baba et al.). This insulin precursor B(1-29)-A(1-21) is prepared by a semisynthetic process from porcine insulin. First the insulin B(1-29) and A(1-21) chains were prepared and coupled to a linear peptide B(1-29)-A(1-21). This compound in the hexathiol form was oxidized in vitro rendering the single chain des-(B30) insulin molecule.

The present invention is based on the surprising discovery that the above single chain insulin precursor B(1-29)-A(1-21) and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)-chain are expressed in high yields and

with correctly positioned disulfide bridges when culturing yeast strains transformed with DNA-sequences encoding such insulin precursors.

According to a first aspect of the present invention there is provided a DNA-sequence encoding insulin precursors of the formula

$$B(1-29)-(X_n-Y)_m-A(1-21) \qquad I$$

wherein X_n is a peptid chain with n amino acid residues, Y is Lys or Arg, n = 0 - 33, m - 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) is the A chain of human insulin with the proviso that the peptid chain $-X_n-Y-$ does not contain two adjacent basic amino acid residues (i.e. Lys and Arg).

Preferred insulin precursors of the above formula I are B(1-29)-A(1-21), i.e. m = 0 in formula I, and compounds with a relative short bridging chain between the B(1-29)- and the A(1-21)- chain.

When m = 1, then n is preferably 1-15, more preferably 1-8 or 1-5 and most preferably 1-3 or 1-2. X may preferably be selected from the group consisting of Ala, Ser and Thr, the individual X's being equal or different. Examples of such preferred compounds are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a second aspect of the present invention there is provided a replicable expression vehicle capable of expression of a DNA-sequence comprising a sequence encoding the insulin precursors of formula I in yeast.

The expression vehicle may be a plasmid capable of replication in the host microorganism or capable of integration into the host organism chromosome. The vehicle employed may code for expression of repeated sequences of the desired DNA-sequence, each separated by selective cleavage sites.

According to a third aspect of the present invention there is provided a process for producing insulin precursors of formula I in yeast wherein a transformant yeast strain including at least one expression vehicle capable of expressing the insulin precursors is cultured in a suitable nutrient medium followed by isolation of the insulin precursors.

According to a fourth aspect of the present invention there are provided novel human insulin precursors. Such novel human insulin precursors have the following general formula

$$B(1-29)-X_n-Y-A(1-21) \qquad II$$

in which the different symbols have the above mentioned definitions. Preferred novel insulin precursors are B(1-29)-Ser-Lys-A(1-21) and B(1-29)-Ala-Ala-Lys-A(1-21).

According to a fifth aspect of the present invention there is provided a yeast strain transformed with an expression vehicle capable of expressing a DNA-sequence comprising a sequence encoding the above insulin precursors in yeast.

The insulin precursors may be expressed with additional protein proceeding the insulin precursor. The additional protein may have the function of protecting the insulin precursor against, e.g. in vivo degradation by endogeneous enzymes or of providing information necessary to transport the desired protein into the periplasmic space and finally across the cell wall into the medium.

The additional protein contains a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors enabling subsequent splitting off the additional protein either by the microorganism itself or by later enzymatical or chemical cleavage.

Accordingly the present invention includes a DNA-sequence encoding the above insulin precursors and further comprising an additional DNA-sequence positioned upstream to the sequence encoding the insulin precursors and encoding an additional amino acid-sequence containing a selective cleavage site adjacent to the N-terminal of the B(1-29)-chain of the insulin precursors.

According to a preferred embodiment of the present invention the additional amino acid sequence comprises at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain of the insulin precursor.

When the insulin precursor is expressed in yeast the additional amino acid-sequence may contain two basic amino acids (e.g. Lys-Lys, Arg-Arg, Lys-Arg or Arg-Lys) adjacent to N-terminal of the B(1-29)-chain of the insulin precursor, yeast being able to cleave the peptide bond between the basic amino acids and the precursor. Also a Glu-Ala or Asp-Ala cleavage site adjacent to the desired protein enables separation of the additional amino acid sequence by the yeast itself by means of a dipeptidase enzyme produced by the yeast.

The insulin precursors may be secreted with an amino acid-sequence linked to the B(1-29)-chain of the precursors provided that this amino acid sequence contains a selective cleavage site adjacent to the B(1-29)-chain for later splitting of the superfluous amino acid sequence. As the insulin precursors do not contain methionine cyanogen bromide cleavage at methionine adjacent to the desired protein would be operative. Likewise, arginine- and lysine-cleavage sites adjacent to the desired protein enables cleavage with trypsinlike proteases.

For secretion purposes the DNA-sequence encoding the insulin precursors may be fused to an additional DNA-sequence coding for a signal peptide. The signal peptide is cleaved off by the transformant microorganism during the secretion of the expressed protein product from the cells ensuring a more simple isolation of the desired product.The secreted product may be the insulin precursor or may contain an additional N-terminal amino acid-sequence to be removed later as explained above.

Secretion may be provided by including in the expression vehicle the yeast MFa1 leader sequence (Kurjan, J. and Herskowitz, I., Cell 30, (1982), 933 - 943) and according to a further preferred embodiment of the present invention the additional amino acid-sequence positioned upstream to the sequence encoding the insulin precursors comprises the yeast MFa1 leader coding sequence or part thereof.

The expression of the desired DNA-sequence will be under control of a promoter sequence correctly positioned to the DNA-sequence encoding the desired protein product to result in expression of the desired protein in the host organism. Preferably a promoter from a gene indigeneous to the host organism may be

used. The DNA-sequence for the desired protein will be followed by a transcription terminator sequence, preferably a terminator sequence from a gene indigeneous to the host organism. If yeast is used as host organism the promoter and terminator sequences are preferably the promoter and terminator of the triose phosphase isomerase (TPI) gene, respectively.

Other promoters may be utilized such as the phosphoglycerate kinase (PGK1)- and the MFα1-promoter.

The present invention further comprises a method for preparing human insulin by which a yeast strain is transformed with a replicable expression vehicle comprising a DNA-sequence encoding the insulin precursors of the above formula I, the transformed yeast strain is cultured in a suitable nutrient medium, the insulin precursors are recovered from the culture medium and converted into human insulin.

The insulin precursors according to the present invention may be converted into mature human insulin by transpeptidation with an L-threonine ester in the presence of trypsin or a trypsin derivative as described in the specification of Danish patent application 574/80 (the disclosure of which is incorporated by reference hereinto) followed by transformation of the threonine ester of human insulin into human insulin by known processes.

If the insulin precursors are secreted with an additional amino acid sequence adjacent to the N-terminal of the B(1-29)-chain such amino acid sequence should either be removed in vitro before the transpeptidation or should contain at least one basic amino acid adjacent to the N-terminal of the B(1-29)-chain as trypsin will cleave the peptide bond between the basic amino acid and the amino group of Phe^{B1} during the transpeptidation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate a preferred embodiment of the present invention.

Fig. 1 illustrates the preparation of plasmid pMT344,
fig. 2 illustrates the preparation of plasmid pMT475,
fig. 3 illustrates the preparation of plasmid pMT212,
fig. 4 illustrates the preparation of plasmid pMT479
fig. 5 illustrates the preparation of plasmid pMT219,
fig. 6 illustrates the preparation of plasmid pMT598,
fig. 7 illustrates the preparation of plasmid pMT610,
fig. 8 illustrates the preparation of plasmid pT5, and
fig. 9 illustrates the preparation of plasmid pMT639.

In the drawings and part of the following description the expression B' is used in stead of B(1-29) and A in stead of A(1-21). Accordingly the expression B'A is equivalent to the expression B(1-29)-A(1-21).

DETAILED DESCRIPTION

1. Preparation of a gene coding for human insulin

Total RNA purified (Chirgwin, J.M. Przybyla, A.E., McDonald, R.J. & Rutter, W.J., Biochemistry 18, (1979) 5294 - 5299) from human pancreas was reverse transcribed (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J.F. & Marcker, K.A., Proc.Natl.Acad.Sci. USA 80, (1983), 2866 - 2869) with AMV reverse transcriptase and d(GCTTTATTCCATCTCTC) as 1. strand primer. After preparative urea-polyacrylamide gel purification of the human insulin cDNA, the second strand was synthesized on this template with DNA polymerase large fragment and d(CAGATCACTGTCC) as primer. After S1 nuclease digestion the human insulin ds. cDNA was purified by polyacrylamide gel electrophoresis, tailed with terminal transferase and cloned in the PstI site on pBR327 (Sorberon et al., Gene 9, (1980), 287 - 305) in E. coli. A correct clone harbouring the plasmid was identified from the recombinants by restriction endonuclease analysis and confirmed by nucleotide sequencing (Maxam, A., & Gilbert, W., Methods in Enzymology, 65 (1980), 499 - 560. Sanger, F., Nicklen, S. & Coulson, A.R., Proc.Natl.Acad.Sci. USA 74, (1977), 5463 - 5467).

2. Preparation of genes coding for precursors of human insulin.

The gene encoding B(1-29)-A(1-21) of human insulin was made by site specific mutagenesis of the human insulin sequence with a 75bp in frame deletion in the C-peptide coding region inserted into a circular single stranded M-13 bacteriophage vector. A modified procedure (K. Norris et al., Nucl.Acids.Res. 11 (1983) 5103 - 5112) was used in which a chemically synthesized 19-mer deletion primer was annealed to the M13 template. After a short enzymatic extension reaction a "universal" 15-mer M13 dideoxy sequencing primer was added followed by enzymatic extension and ligation. A double stranded restriction fragment (BamH1-Hind III) was cut out of the partly single stranded circular DNA and ligated into pBR322 cut with BamHI and Hind III.

The obtained ligation mixture was used to transform E. coli and transformants harbouring a plasmid pMT319 containing the gene encoding B(1-29)-A(1-21) of human insulin was identified.

Genes encoding B(1-29)-Ala-Ala-Lys-A(1-21) and B(1-29)-Ser-Lys-A(1-21) were made accordingly by insertion of a fragment encoding MFα1-B-C-A in the M-13 bacteriophage and site specific mutagenesis of the human insulin sequence with chemically synthesized 30-mer and 27-mer deletion primers, respectively, and the above mentioned "universal" 15-mer M13 dideoxy sequencing primer. A double stranded restriction fragment (Xbal-EcoR1) was cut out of the partly single stranded circular DNA and ligated into pUC13 and pT5, respectively. By transformation and retransformation of E. coli transformants harbouring a plasmid pMT598 containing the gene encoding B(1-29)-Ala-Ala-Lys-A(1-21) and pMT630 containing the gene encoding B(1-29)-Ser-Lys-A(1-21) were identified.

A gene encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was made in a similar way as described above by insertion of a fragment encoding MFα1-B(1-29)-A(1-21) in a M13 mp11 bacteriophage and site specific mutagenesis of the B(1-29)-A(1-21) sequence with a chemically synthesized 46-mer deletion primer (5'-CACACCCAAGACTAAAGAAGCTGAAGACTTGCAAAGAGGCATTGTG-3') and the "universal" primer. Also, by a similar procedure a gene

encoding B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-A(1-21) was constructed.

3. Plasmid constructions.

The gene encoding B(1-29)-A(1-21) of human insulin (B'A) was isolated as a restriction fragment from pMT319 and combined with fragments coding for the TPI promoter (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434), the MFα1 leader sequence (J. Kurjan and I. Herskowitz,. Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) and the transcription termination sequence from TPI of S.cerevisiae. These fragments provide sequences to ensure a high rate of transcription for the B'A encoding gene and also provide a presequence which can effect the localization of B'A into the secretory pathway and its eventual excretion into the growth medium. This expression unit for B'A ($TPI_{promoter}$-MFα1 leader - B'A - $TPI_{terminator}$) was then placed on a plasmid vector containing the yeast 2μ origin of replication and a selectable marker, LEU 2, to give pMT344, a yeast expression vector for B'A.

During in vivo maturation of α-factor in yeast, the last (C-terminal) six amino acids of the MFα1 leader peptide (Lys-Arg-Glu-Ala-Glu-Ala) are removed from the α-factor precursor by the sequential action of an endopeptidase recognizing the Lys-Arg sequence and an aminodipeptidase which removes the Glu-Ala residues (Julius, D. et al. Cell 32 (1983) 839 - 852). To eliminate the need for the yeast aminodipeptidase, the sequence coding for the C-terminal Glu-Ala-Glu-Ala of the MFα1 leader was removed from pMT344 via in vitro mutagenesis. The resulting yeast expression plasmid, pMT475, contains the insert coding for $TPI_{promotor}$-MFα1 leader (minus Glu-Ala-Glu-Ala) - B'A - $TPI_{terminator}$.

In a preferred construction the modified expression unit was transferred to a stable, high copy number yeast plasmid CPOT, (ATCC No. 39685), which can be selected merely by the presence of glucose in the growth medium. The resulting yeast expression vector for B'A was numbered pMT479.

The fragment encoding MFa1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21) was isolated as a restriction fragment from pMT598 and combined with fragments coding for the TPI promoter and the TPI terminator and transferred to the above mentioned high copy number yeast plasmid CPOT. The resulting yeast expression vector for B(1-29)-Ala-Ala-Lys-A(1-21) was numbered pMT610.

The fragment containing the insert TPI_P- MFa1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPI_T was isolated as a restriction fragment from pMT630 and transferred into CPOT. The resulting yeast expression vector for B(1-29)-Ser-Lys-A(1-21) was numbered pMT639.

The fragment containing the insert TPI_P- MFa1 leader-(minus Glu-Ala-Glu-Ala)-B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21)-TPI_T was inserted into a high copy number yeast plasmid DPOT, being a CPOT derivative containing a SphI-BamHI-fragment of pBR322 inserted into a SpHI-BamHI fragment of CPOT. The resulting yeast expression vector for B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21) was numbered p1126.

4. Transformation

Plasmids pMT344 and pMT475 were transformed into S. cerevisiae leu 2 mutants by selection for leucin prototrophy as described by Hinnen et al.(A. Hinnen, J.B. Hicks and G.R. Fink. Transformation of Yeast. Proc.Nat.Aca.Sci. 75 (1978) 1929).

Plasmids pMT479, pMT610, pMT639 and p1126 were transformed into S. cerevisiae strains carrying deletions in the TPI gene by selecting for growth on glucose. Such strains are normally unable to grow on glucose as the sole carbon source and grows very slowly on galactose lactate medium. This defect is due to a mutation in the triose phosphate isomerase gene, obtained by deletion and replacement of a major part of this gene with the S.

cerevisiae LEU 2 gene. Because of the growth deficiencies there is a strong selection for a plasmid which contains a gene coding for TPI. pMT479 contains the Schizo. pombe TPI gene.

5. Expression of human insulin precursors in yeast

Expression products of human insulin type were measured by radioimmunoassay for insulin as described by Heding, L. (Diabetologia 8, 260 - 66, 1972) with the only exception that the insulin precursor standard in question was used instead of an insulin standard. The purity of the standards were about 98% as determined by HPLC and the actual concentration of peptide in the standard was determined by amino acid analysis. The expression levels of immunoreactive human insulin precursors in the transformed yeast strains are summarized in Table 1.

Table 1

Expression levels of immunoreactive human insulin precursors in yeast.

Yeast strain	Plasmid	Construct	Immunoreactive insulin precursor (nmol/l supernatant)
MT 350 (DSM 2957)	pMT 344	B(1-29)-A(1-21)	100
MT 371 (DSM 2958)	pMT 475	B(1-29)-A(1-21)	192
MT 519 (DSM 2959)	pMT 479	B(1-29)-A(1-21)	2900
MT 620 (DSM 3196)	pMT 610	B(1-29)-Ala-Ala-Lys-A(1-21)	1200 - 1600
MT 649 (DSM 3197)	pMT 639	B(1-29)-Ser-Lys-A(1-21)	1600
E18 # 9	p1126	B(1-29)-Thr-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-A(1-21)	200

The isolation and characterization of expression products are given in Examples 7 - 9 and 12 - 13.

6. Conversion of human insulin precursor into B30 esters of human insulin

The conversion of the human insulin precursors into human insulin esters can be followed quantitatively by HPLC (high pressure liquid chromatography) on reverse phase. A 4 x 300 mm "μBondapak C18 column" (Waters Ass.) was used and the elution was performed with a buffer comprising 0.2 M ammonium sulphate (adjusted to a pH value of 3.5 with sulphuric acid) and containing 26 - 50% acetonitrile. The optimal acetonitrile concentration depends on which ester one desires to separate from the insulin precursor. In case of human insulin methyl ester separation is achieved in about 26% (v/v) of acetonitrile.

Before the application on the HPLC column the proteins in the reaction mixture were precipitated by addition of 10 volumes of acetone. The precipitate was isolated by centrifugation, dried in vacuo, and dissolved in 1 M acetic acid.

EXPERIMENTAL PART

Example 1

Construction of a gene coding for B(1-29)-A(1-21)insulin

Materials and Methods

15-mer M13 dideoxy sequencing primer d(TCCCAGTCACGACGT), T4 DNA ligase and restriction enzymes were obtained from New England Biolabs. DNA polymerase I "Klenow fragment" and T_4 polynucleotide kinase were purchased from P-L Biochemicals. (γ-^{32}P)-ATP (7500 Ci/mmol) was obtained from New England Nuclear. The support for oligonucleotide synthesis was 5'-O-dimethoxytrityl N^2-isobutyryldeoxyguanosine bound via a 3'-O-succinyl group to aminomethylated 1% crosslinked polystyrene beads from Bachem.

Construction of M13 mp10 insHXΔPst phage:

The M13 mp10 derived phage mp10 insHX was constructed by cloning of the 284 bp large proinsulin coding Hind III-XbaI fragment, isolated from p285, into Hind III-XbaI cut M13 mp10 RF (Messing, J. and Vieira, J. (1983) Unpublished results).

M13 mp10 insHXΔPst was constructed from mp10 insHX,RF by complete PstI digestion followed by ligation and transformation of E. coli JM103. The resulting phage harbours the human proinsulin coding sequences, with a 75 bp in frame deletion in the C-peptide coding region. Single stranded phage was prepared as described (Messing, J. and Vieira, J. (1982) Gene 19, 269 - 276).

Oligodeoxyribonucleotide synthesis

The 19-mer deletion primer d(CACACCCAAGGGCATTGTG) was synthesized by the triester method on a 1% crosslinked polystyrene support (Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) Nucl.Acids Res. 10, 1755 - 1769). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an HPLC pump and a control module. The oligonucleotide was purified after deprotection by HPLC on a LiChrosorb RP18 column (Chrompack (Fritz, H.-J., Belagaje, R., Brown, E.L., Fritz, R.H., Jones, R.A., Lees, R.G., and Khorana, H.G. (1978) Biochemistry 17, 1257 - 1267).

5'-^{32}P-labelling of oligodeoxyribonucleotide

The 19-mer was labelled at the 5'end in a 60µl reaction mixture containing 50 mM Tris-HCl at pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 0.4% glycerol, 120 pmole ATP, 50 µCi of (γ-^{32}P)-ATP (10 pmole), 120 pmole of oligonucleotide and 30 units of T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min., and terminated by heating at 100°C for 3 min. The labelled oligonucleotide was separated from unreacted (γ-^{32}P)-ATP by chromatography on a column (1 x 8 cm) of Sephadex G50 superfine in 0.05 M triethylammonium bicarbonate at pH 7.5.

For colony hybridization the oligonucleotide was labelled without the addition of "cold" ATP as described (Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J., and Marcker, K. (1983) Proc.Natl.Acad.Sci. USA 80, 2866 - 2869).

Oligodeoxyribonucleotide primed DNA synthesis

Single stranded M13 mp10 insHXΔPst (0.4 pmole was incubated with the 19-mer 5'-(^{32}P)-labelled oligodeoxyribonucleotide primer (10 pmole) in 20 µl of 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DDT for 5 min. at 55°C and annealed for 30 min. at 11°C. Then 9 µl of d-NTP-mix consisting of 2.2 mM of each dATP, dCTP, dGTP, dTTP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DDT was added followed by 7 units of E. coli DNA polymerase I (Klenow). The mixture was kept for 30 min. at 11°C and heated for 10 min. at 65°C. 15-mer universal primer for dideoxy sequencing (4 pmole) was added and the mixture heated at 65°C for an additional minute. After cooling to 11°C 26 µl of solution containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.8 mM of each dATP, dCTP, dGTP, dTTP, 2.4 mM ATP and 10^3 units of T4 ligase was added followed by 9.5 units of E. coli DNA polymerase I (Klenow). The final volume of the mixture was 64 µl. After incubation for 3 hours at 11°C 20 µl 4M sodium acetate was added, and the volume adjusted to 200 µl with TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The mixture was extracted twice with phenol/chloroform. 0.9 µg (0.3 pmole) of the purified large fragment of pBR322 cleaved with BamHI and Hind III was added as carrier DNA. After ether extraction of the aqueous phase, the DNA was isolated by ethanol precipitation.

Endonuclease digestion

The DNA, prepared as described above, was digested respectively with 16 and 20 units of restriction endonucleases BamHI and Hind III in a total volume of 22µl of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DDT, 4 mM spermidine). The mixture was extracted with phenol/chloroform

followed by ether and the DNA was isolated by ethanol precipitation and then dissolved in 12 µl H_2O. 2µl was used for electrophoresis on a 7M urea 6% polyacrylamide gel.

Ligation

To a part of the DNA (5 µl) was added a new portion of the purified large fragment of pBR322 cut with BamHI and Hind III (0.38 µg) and 400 units of T4 DNA ligase, in a total volume of 41 µl containing 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DDT, 40 µg/ml gelatine. Ligation was performed at 16°C for 16 hours.

Transformation

20.5 µl of the ligation mixture was used to transform $CaCl_2$ treated E. coli MC 1000 (r^-, m^+). The bacteria were plated on LB-agar plates and selected for resistance to ampicillin (100 µg/ml). 2.6 x 10^3 colonies per pmole of M13 mp10 insHX Pst were obtained.

Colony hybridisation

123 transformed colonies were picked onto fresh ampicillin plates and grown overnight at 37°C. Colonies were transferred to Whatman 540 filter paper and fixed (Gergen, J.P., Stern, R.H., and Wensink, P.C. (1979), Nucl.Acids Res. 7, 2115 - 2136). A prehybridization was performed in a sealed plastic bag with 6 ml of 0.9 M NaCl, 0.09 M Tris-HCl pH 7.5 0.006 M EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1% SDS and 50 µg/ml salmon sperm DNA for 2 hours at 65°C. Then 8.5 x 10^6 cpm of ^{32}P-labelled 19-mer was added and hybridisation performed at 45°C overnight. The filter was washed with 0.9 M NaCl, 0.09 M sodium citrate three times at 0°C for 5 min. and was then autoradiographed and washed once at 45°C for 1 min. and autoradiographed again. After washing at 45°C, identification of 3 colonies containing mutated plasmid was possible.

Endonuclease analysis of mutated plasmids

Plasmids from the supposed mutant colonies were prepared by a rapid method (Ish-Horowicz, D. and Burke, J.F. (1981), Nucl.Acids Res. 9, 2989 - 2998), digested with a mixture of BamHI and Hind III and then analysed by electrophoresis on a 2% agarose gel. The presence of a 179 bp fragment confirmed that the 3 colonies contained mutant plasmid.

Retransformation

The colonies identified as "mutant" contain plasmids which are the progeny of a heteroduplex. Pure mutant could be obtained by retransformation of $CaCl_2$ treated E. coli MC1000 (r^-, m^+)with plasmid from 2 of the mutant colonies. From each plate 5 ampicillin resistant clones were isolated, plasmid DNA was prepared and analysed by endonuclease cleavage as mentioned above. 3 out of 5 and 5 out of 5 respectively were shown to be pure mutant. One plasmid pMT319 was selected for further use.

DNA sequence analysis

5 µg of pMT319 was cleaved with BamHI under standard conditions, phenol extracted and ethanol precipitated. Filling in of the BamHI sticky ends was performed with Klenow DNA polymerase I, dCTP, dGTP, dTTP, and α-^{32}P-dATP.

After phenol extraction and ethanol precipitation the DNA was digested with EcoRI. The ^{32}-P labelled fragment with the deletion was purified by electrophoresis on a 2% agarose gel and sequenced by the Maxam-Gilbert method (Maxam, A. and Gilbert, W. (1980) Methods in Enzymology 65, 499 - 560).

Example 2

Construction of a yeast plasmid pMT344 for expression of B(1-29)-A(1-21) of human insulin (B'A).

Plasmid pMT319 containing the gene coding for B'A and constructed as explained above was cut with restriction enzymes Hind III and XbaI and a 0.18 kb fragment was isolated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold

Spring Harbor Press 1982) from a 2% agarose gel. Similarly a fragment (6.5 kb XhoI - Hind III) containing the S. cerevisiae TPI promotor (T. Alber and G. Kawasaki. Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434) and the MFα1 leader sequence (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) was isolated from plasmid p285 constructed as described in US-patent application S.N. 547,748 of November 1, 1983. A fragment (0.7 kb XbaI - BamHI) containing the TPI transcription termination sequences (T. Alber and G. Kawasaki, Nucleotide Sequence of the Triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. 1 (1982) 419 - 434) was also isolated from p285. Finally a 5.4 kb XhoI - BamHI fragment was isolated from the yeast vector YEp13 (J.R. Broach. Construction of High Copy Yeast Vectors Using 2μm Circle Sequences. Methods Enzymology 101 (1983) 307 - 325). The above four fragments were ligated (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) and transformed into E. coli (T. Maniatis, E.F. Fritsch, and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press 1982) selecting for ampicillin resistance. Plasmids were isolated from the transformants and the structure of one of these, pMT344, verified by restriction mapping. The construction and main features of pMT344 are outlined in fig. 1.

Example 3

Construction of a yeast plasmid pMT475 for expression of B(1-29)-A(1-21) of human insulin (B'A) after a modified MFα1 leader.

To construct a plasmid for the expression of B'A after a MFα1 leader (J. Kurjan and I. Herskowitz, Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains four Tandem Copies of Mature α-Factor. Cell 30 (1982) 933 - 943) lacking its last four amino acids (Glu Ala Glu Ala), the 0.14 kb XbaI - EcoRII fragment containing the A and part of the B' sequences was isolated from pMT319. Likewise the 5' proximal part

of the B' gene was isolated as a 0.36 kb EcoRI - EcoRII fragment from pM215. This plasmid was constructed by subcloning the EcoRI - XbaI fragment containing the insulin BCA gene from p285 into pUC13 (constructed as described for pUC8 and pUC9 by Vieira et al., Gene 19: 259 - 268 (1982)) and subsequent in vitro loop-out removal of the 12 bases coding for Glu Ala Glu Ala at the junction between MFa1 leader and insulin BCA. These two pieces covering the B'A gene were ligated to EcoRI - XbaI digested pUC13 vector (see fig. 2) to give pMT473. The modified gene contained within a 0.5 kb EcoRI - XbaI fragment was isolated from pMT473 and then ligated to two fragments (4.3 kb XbaI - EcoRV and 3.3 kb EcoRV - EcoRI) from pMT342. pMT342 is the yeast vector pMT212 with an inserted $TPI_{promotor}$-MFa1 leader - BCA - $TPI_{terminator}$. The resulting plasmid, pMT475, contains the insert: $TPI_{promotor}$ - MFa1 leader (minus Glu-Ala-Glu-Ala) - B'A - $TPI_{terminator}$. The construction of plasmids pMT342, 473 and 475 is outlined in fig. 2. The construction of the vector pMT212 is shown in fig. 3. Plasmid pMLB1034 is described by M.L. Berman et al., Advanced Bacterial Genetics, Cold Spring Harbor (1982), 49 - 51.

Example 4

Insertion of the B(1-29)-A(1-21) (B'A) gene into a stable yeast plasmid pMT479.

The modified B'A gene from pMT475 was isolated as a 2.1 kb BamHI - partial SphI fragment and ligated to an approximately 11 kb BamHI - SphI fragment of plasmid CPOT to give plasmid pMT479 (fig. 4). Plasmid CPOT is based on the vector C1/1 which has been modified by substituting the original pBR322 BglI - BamHI fragment with the similar BglI - BamHI fragment from pUC13 and subsequent insertion of the S.pombe TPI gene (US patent application S.N. 614,734 filed on May 25, 1984) as a BamHI - SalI fragment to give CPOT. C1/1 is derived from pJDB 248, Beggs et al., Nature 275, 104 - 109 (1978) as described in US patent application S.N. 489,406, filed April 28, 1983).

Example 5

Transformation

S. cerevisae strain MT118 (a, leu 2, ura 3, trp 1) was grown on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) to an $O.D._{600}$ of 2.1. 100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of (1.2 M sorbitol, 25 mM Na_2EDTA pH= 8.0, 6.7 mg/ml dithiotreitol). The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of (1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate pH = 5.8, 2 mg Novozym® 234). The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and in 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (Tris = Tris(hydroxymethyl)-aminometan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of plasmid pMT344 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) with leucine omitted and containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant MT350 (=MT 118/pMT344) was chosen for further characterization.

Plasmid pMT475 was transformed into S.cerevisiae strain MT 362 (α,leu2) by the same procedure as above, and the transformant MT371 (=MT362/pMT475) isolated.

Transformation of pMT479 into strain E2-7B X E11-3C (a/α, Δtpi/Δtpi, pep 4-3/pep 4-3; this strain will be referred to as MT501) was performed as above with the following modifica-

tions: 1) prior to transformation strain MT501 was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an $O.D._{600}$ of 0.6. 2) the SOS solution contained YPGaL instead of YPD. One transformant MT519 (=MT501/pMT479) was chosen for further characterization.

The transformed microorganisms MT 350, MT 371 and MT 519 were deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), Griesebachstrasse 8, D-3400 Göttingen, on May 15, 1984 and accorded the reference numbers DSM 2957, DSM 2958, and DSM 2959, respectively.

Example 6

Expression of B(1-29)-A(1-21) insulin in yeast

Strains MT350 (DSM 2957) and MT371 (DSM 2958) were grown in synthetic complete medium SC (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981) with leucine omitted. For each strain, two 1 liter cultures in 2 liter baffled flasks were shaken at 30°C until they reached $O.D._{600nm}$ of 7 to 10. They were then centrifuged and the supernatant removed for further analysis.

Strain MT519 (DSM 2959) was grown similarly but on YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) and to an $O.D._{600nm}$ of 15, centrifuged and the supernatant separated for analysis as above.

Example 7

Expression of B(1-29)-A(1-21) insulin in yeast strain MT350 (DSM 2957)

Yeast strain MT350 (DSM 2957) was grown as previously described in example 6 and expression products from 1100 ml of supernatant from this strain were isolated as follows:

10 g of LiChroprep® RP-18 (Merck, art. 9303) were washed 3 times with 50 mM NH_4HCO_3, 60% EtOH and thereafter packed in a 6 x 1 cm column. The column was equilibrated with 50 ml of 50 mM NH_4HCO_3. 55 ml of 96% EtOH were added to 1100 ml of the yeast supernatant, and the mixture was applied to the column overnight (flow: 70 ml/h).

The column was washed with 10 ml of 0.5 M NaCl and 10 ml of H_2O, and the peptides were eluted with 50 mM of NH_4HCO_3, 60% EtOH. The eluate (5 ml) was concentrated by vacuum centrifugation to 1.4 ml (to remove the ethanol), and the volume was adjusted to 10 ml with 25 mM of HEPES buffer pH = 7.4. The sample was applied to an antiinsulin immunoabsorption column (AIS column) (2.5 x 4.5 cm) which had been washed 4 times with 5 ml of NaPAM-buffer (Heding, L., Diabetologia 8, 260-66, 1972) and twice with 5 ml of 25 mM HEPES-buffer prior to the application. After the application, the column was allowed to stand for 30 min. at room temperature and was thereafter washed 10 times with 4 ml of 25 mM HEPES buffer. The peptides were eluted with 20% HAc. The pH value of the eluate was adjusted to 7.0 with NH_4OH, and the pool was concentrated to 500 µl by vacuum rotation.

The sample from the previous step was further purified on HPLC on a 10µ Waters µBondopak C-18 column (3.9 x 300 mm). The A and B buffers were 0.1% TFA in H_2O and 0.07% TFA in MeCN, respectively. The column was equilibrated with 25% B (flow: 1.5 ml/min.) and the peptides were eluted with a linear gradient of MeCN (1%/min.) and detected at 276 nm. The yield in each step of the purification was determined by radioimmunoassay as previously described, and Table 2 summarizes the purification. The overall yield was 68%.

Table 2

Purification of expression products from yeast strain MT350 supernatant

Purification step	Volume (ml)	Immunoreactive B(1-29)-A(1-21) insulin (nmol)
Supernatant	1100	110[x]
RP-18	10	116
Anti-insulin Sepharose	0.5	116
HPLC	2.5	75

x) Dilution effect was observed in this sample

Only one peak containing immunoreactive B(1-29)-A(1-21) insulin material was detected from the HPLC column. Peptide material from this peak was isolated and subjected to amino acid sequence analysis. The sequence analysis was performed with a Gas Phase sequencer (Applied Biosystem Model 470A) as described by Hewick, R.M. et al. (J.Biol.Chem. 256, 7990-7997, 1981). From the sequencing results it could be concluded that the expression products consisted of 3 peptides:

$(Glu-Ala)_2$-B(1-29)-A(1-21) insulin	89%
Glu-Ala-B(1-29)-A(1-21) insulin	2%
B(1-29)-A(1-21) insulin	9%

The peptides were present in the relative amount as indicated.

Example 8

Expression of B(1-29)-A(1-21) insulin in yeast strain MT371 (DSM 2958)

Yeast strain MT371 (DSM 2958) was grown as previously described in example 6 and expression products from 665 ml of supernatant from this strain were isolated as described in Example 7. The overall yield was 50 nmol, corresponding to 39%. Peptide material was isolated from the HPLC column and sequenced as described in Example 7. From the sequence results (18 residues from the N-terminal) it could be concluded that the peptide was homogeneous B(1-29)-A(1-21) insulin.

Example 9

Expression of B(1-29)-A(1-21) insulin in yeast strain MT519 (DSM 2959)

Yeast strain MT519 (DSM 2959) was grown as previously described in example 6 and expression products from 70 ml of supernatant were isolated as described in example 7. The overall yield was 116 nmol, corresponding to 57%. The peptide was sequenced as described in Example 7. As judged from the 42

residues identified from the N-terminal end, the peptide was homogeneous B(1-29)-A(1-21) insulin. Approximately 5 nmol of peptide was hydrolyzed in 100 µl 6N HCl for 24 h at 110°C. The hydrolysate was analyzed on a Beckman Model 121M amino acid analyser. The following amino acid composition was found:

Table 3

Amino acid analysis of purified B(1-29)-A(1-21) insulin

Amino acid	Found	Theory	Amino acid	Found	Theory
Asx*	2.97	3	Val	3.37	4
Thr	1.77	2	Ile	1.65	2
Ser	2.45	3	Leu*	5.65	6
Glx*	6.68	7	Tyr	3.51	4
Pro	1.33	1	Phe*	2.73	3
Gly*	3.95	4	Lys*	0.95	1
Ala*	1.22	1	His*	1.84	2
Cys 0.5	4.54	6	Arg*	1.13	1

*) amino acid used for normalization.

Example 10

Construction of a yeast plasmid pMT610 for expression of B(1-29)-Ala-Ala-Lys-A(1-21)

A 4.3 kb EcoRI-XbaI and a 3.3 kb EcoRI-EcoRV fragment from pMT342 (see example 3) were ligated to a 0.6 kb EcoRI-XbaI fragment of pM215 (see example 3). The obtained plasmid pMT462 harbours the insert MFa1 leader (minus Glu-Ala-Glu-Ala)-B-C-A. For converting the B-C-A encoding fragment into a B(1-29)-Ala-Ala-Lys-A(1-21) encoding fragment the modified site specific mutagenesis procedure (K. Norris et al., ibid.) was used. A 0.6 kb EcoRI-XbaI fragment from pMT462 encoding MFa1 leader (minus Glu-Ala-Glu-Ala)-B-C-A was inserted into M13 mp10 RF phage cut with XbaI-EcoRI. Single strand M13 phage containing the above

EcoRI-Xbal insert was incubated with a 30mer d(TTCACAATGCCCTTAGCGGCCTTGGGTGTG) primer (KFN15) and the "universal" 15-mer M13 primer d(TCCCAGTCACGACGT) (see example 1), heated to 90°C for 5 minutes and slowly cooled to room temperature in order to allow annealing. Then partly double stranded DNA was made by addition of a d-NTP-mix, Klenow Polymerase and T4 ligase.After phenol extraction, ethanol precipitation and resuspension, the DNA was cut with restriction enzymes Apal,Xbal and EcoRl. After another phenol extraction, ethanol precipitation and resuspension, the DNA was ligated to EcoRl-Xbal cut pUC13. The ligation mix was transformed into an E.coli (r^-m^+) strain and plasmids were prepared from a number of transformants. Plasmid preparations were cut with EcoRl and Xbal and those preparations showing bands at both 0.5 and 0.6 kb were retransformed into E.coli. From the retransformation a transformant harbouring only pUC13 with a 0.5 kb insert was selected. The sequence of the EcoRl-Xbal insert of this plasmid, pMT598, was then confirmed by the Maxam-Gilbert method to encode MFα1 leader (minus Glu-Ala-glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21). The Xbal-EcoRI insert from pMT598 was provided with TPI promotor and TPI terminator by ligation of a 0.5 kb Xbal-EcoRI fragment of pMT598 with a 5.5 kb Xbal-EcoRI fragment of pT5. The construction of pT5 harbouring the insert TPIp-MFα1 leader-BCA-TPI_T is illustrated in fig. 8. The resulting plasmid pMT 601 containing the insert TPIp-MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ala-Ala-Lys-A(1-21)-TPI_T was cut with BamHl and partially with Sphl and the 2.1 kb fragment was inserted in CPOT cut with BamHI and Sphl. The resulting plasmid pMT610 was used for transformation of yeast.

Example 11

Construction of a yeast plasmid pMT639 for expression of B(1-29)-Ser-Lys-A(1-21)

The BCA encoding fragment from pMT462 (see example 10) was converted into B(1-29)-Ser-Lys-A(1-21) by a procedure analogous with the procedure described in example 10 by site specific mutagenesis with a mixture of a 27-mer d(TCCACAATGCCCTTAGACTTGGGTGTG) primer KFN36 and the "universal"

15-mer M13 primer. After filling in with Klenow polymerase and ligation with T4 ligase the partly double stranded DNA was digested with ApaI, EcoRI and XbaI and ligated with the 5.5 kb XbaI - EcoRI fragment from plasmid pT5 (see example 10). After transformation and retransformation into E.coli, a plasmid pMT 630 containing the insert MFα1 leader (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21) was isolated and the sequence of the insert confirmed. The further procedure for obtaining plasmid pMT639 containing the insert TPIp-MFα1 (minus Glu-Ala-Glu-Ala)-B(1-29)-Ser-Lys-A(1-21)-TPI_T was as described in example 10. The construction of pMT639 is illustrated in Fig. 9.

Example 12

Expression of B(1-29)-Ala-Ala-Lys-A(1-21) in yeast strain MT 620

S. cerevisiae strain MT501 (see example 5) was transformed with pMT 610 as described for pMT479 in example 5. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant MT 620 = (MT501/pMT610) was chosen for further characterization. MT620 was deposited by the applicant with Deutsche Sammlung von Mikroorganismen (DSM), on January 16, 1985 and accorded the reference number DSM 3196.

MT 620 was grown on YPD medium. A two liter culture in 2 liter baffled flask was shaken at 30°C to an $O.D._{600nm}$ of 15. After centrifugation the supernatant was removed for further analysis. The expression level determined by radioimmunoassay was 1.2 µmol/l. Expression products from 840 ml of supernatant were purified as described in Example 7. (RP-18 column, Anti-insulin Sepharose and HPLC). The overall yield was 100 nmol corresponding to about 10%. Peptide material was isolated from the HPLC-column and sequenced as described in Example 7. 35 Edman degradation cycles were carried out (Table 4). From the sequence results the position of the 3 amino acid residue chains (Ala-Ala-Lys) separating the B(1-29) and the A(1-21) chains was confirmed (see table 4).

Table 4

Sequence analysis of B(1-29)-Ala-Ala-Lys-A(1-21) isolated from the culture medium of strain MT 620.

Cyclus No.	PTH-amino acid residue	Yield (pmol)
1	Phe	3361
2	Val	1738
3	Asn	5169
4	Gln	2750
5	His	2045
6	Leu	1405
7	Cys	-
8	Gly	1372
9	Ser	345
10	His	1105
11	Leu	2228
12	Val	1963
13	Glu	1219
14	Ala	1514
15	Leu	1793
16	Tyr	1707
17	Leu	1354
18	Val	1765
19	Cys	-
20	Gly	882
21	Glu	1019
22	Arg	1100
23	Gly	1123
24	Phe	1492
25	Phe	2042
26	Tyr	1014
27	Thr	195
28	Pro	710
29	B_{29}Lys	1173
30	Ala	1026
31	Ala	885
32	Lys	1175
33	A_1Gly	552
34	Ile	518
35	Val	548

The average repetitive yield was 95.6%.

Example 13

Expression of B(1-29)-Ser-Lys-A(1-21) in yeast strain MT643

S. cerevisiae strain MT501 was transformed with pMT639 as described for pMT479 in example 5.

One transformant MT643 = (MT501/pMT639) was chosen for further characterization. MT643 was deposited by the applicant at DSM on January 16, 1985 and accorded the reference No. DSM 3197.

MT643 was grown as described in example 12. After centrifugation the supernatant was removed for further analysis.

The expression level of the insulin precursor determined by radioimmunoassay was 1.6 µmol/l. Expression products from the supernatant from strain MT 643 was isolated as described in Example 7. The peptide material isolated from the HPLC column was submitted to sequence analysis as described in Example 7. From the sequence results (not shown) the position of the two amino acid residues chains (Ser-Lys) separating the B(1-29) and A(1-21) chains was confirmed.

Example 14

Conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10 M acetic acid. 0.26 ml of 1.54 M Thr(Bu^t)-OBu^t in N,N-dimethylacetamide was added. The mixture was cooled to 12°C. 2.8 mg of trypsin dissolved in 0.035 ml of 0.05 M calcium acetate was added. After 72 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr(Bu^t)-OBu^t(B30) human insulin was 64% by HPLC.

Example 15

Conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin

20 mg of B(1-29)-A(1-21) was dissolved in 0.1 ml of 10 M acetic acid. 0.26 ml of 1.54 M Thr-OMe in a mixture of dimethyl sulphoxide and butane-1,4 diol 1/1 (v/v) was added. 1 mg of lysyl endopeptidase from Achromobacter lyticus (Wako Pure Chemical Industries, Osaka, Japan) in 0.07 ml of water was added. After 120 hours at 25°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation, and dried in vacuo. The conversion of B(1-29)-A(1-21) to Thr-OMe(B30) human insulin was 75% by HPLC.

Example 16

Conversion of B(1-29)-Ser Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg of B(1-29)-Ser-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N-dimethylformamide (v/v) in water. 0.2 ml of 2 M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12°C. 2 mg of trypsin in 0.05 ml 0.05 M calcium acetate was added. After 24 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ser-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 95% by HPLC.

Example 17

Conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin

20 mg of B(1-29)-Ala-Ala-Lys-A(1-21) was dissolved in 0.1 ml of a mixture of 34.3% acetic acid (v/v) and 42.2% N,N dimethylformamide (v/v) in water. 0.2 ml of 2 M Thr-OBu^t as hydroacetate salt in N,N-dimethylformamide was added. The mixture was thermostated at 12°C. 2 mg of trypsin in 0.05 ml 0.05 M calcium acetate was added. After 96 hours at 12°C, the proteins were precipitated by addition of 4 ml of acetone, isolated by centrifugation and dried in vacuo. The conversion of B(1-29)-Ala-Ala-Lys-A(1-21) to Thr-OBu^t(B30) human insulin was 84% by HPLC.

Example 18

Preparation of human insulin from various human insulin esters

The human insulin esters in the crude acetone precipitates were purified by gelfiltration and anion exchange chromatography as described in Methods in Diabetes Research vol.1, p. 407 - 403 (Eds. J. Larner & S. Pohl (John Wiley Sons, New York, 1984)). The method was applicable to any of the 3 human insulin esters. The cleavages of the various ester groups, rendering human insulin in nearly 100% yields, were carried out

by hydrolysis of Thr-OMe(B30) human insulin and by acidolysis with trifluoroacetic acid of Thr(Bu^t)-OBu^t(B30) human insulin and of Thr-OBu^t(B30) human insulin as described ibid. p. 409.

CLAIMS

1. A DNA-sequence comprising a sequence encoding an insulin precursor containing a peptide chain with the formula

$$B(1\text{-}29)\text{-}(X_n\text{-}Y)_m\text{-}A(1\text{-}21) \qquad I,$$

wherein X_n is a peptide chain with n amino acid residues, Y is Lys or Arg, n = 0 - 33, m = 0 or 1, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) is the A chain of human insulin, with the proviso that the peptide chain $-X_n-Y-$ does not contain two adjacent basic amino acid residues.

2. A DNA-sequence according to claim 1 wherein m is 0.

3. A DNA-sequence according to claim 1 wherein m is 1 and n is 1 - 8.

4. A DNA-sequence according to claim 1 wherein the peptide chain is

B(1-29)-Ala-Ala-Lys-A(1-21).

5. A DNA-sequence according to claim 1 wherein the peptide chain is B(1-29)-Ser-Lys-A(1-21).

6. A replicable expression vehicle capable of expressing a DNA-sequence according to claims 1 to 5 in yeast.

7. A replicable expression vehicle according to claim 6 comprising the plasmid pMT344 illustrated in Fig. 1 hereof.

8. A replicable expression vehicle according to claim 6 comprising the plasmid pMT475 illustrated in Fig. 2 hereof.

9. A replicable expression vehicle according to claim 6 comprising the plasmid pMT 479 illustrated in Fig. 4 hereof.

10. A replicable expression vehicle according to claim 6 comprising the plasmid pMT610.

11. A replicable expression vehicle according to claim 6 comprising the plasmid pMT639.

12. A yeast strain transformed with an expression vehicle according to claims 6 - 11

13. A process for producing an insulin precursor of formula I according to claim 1 wherein a transformant yeast strain including a replicable expression vehicle according to claims 6 - 11 is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

14. A process for producing an insulin precursor with the formula B(1-29)-A(1-21) wherein a yeast strain DSM 2957 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

15. A process for producing an insulin precursor with the formula B(1-29)-A(1-21) wherein a yeast strain DSM 2958 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

16. A process for producing an insulin precursor with the formula B(1-29)-A(1-21) wherein a yeast strain DSM 2959 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

17. A process for producing an insulin precursor with the formula B(1-29)-Ala-Ala-Lys-A(1-21) wherein a yeast strain DSM 3196 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

18. A process for producing an insulin precursor with the formula B(1-29)-Ser-Lys-A(1-21) wherein a yeast strain DSM 3197 or a variant or mutant thereof productive of the insulin precursor is cultured in a suitable nutrient medium followed by recovery of the insulin precursor.

19. A process for preparing human insulin wherein a yeast strain is transformed with a replicable expression vehicle according to claims 6 - 11, the transformed yeast strain is cultured in a suitable nutrient medium, the expressed insulin precursor is recovered from the culture medium and converted into human insulin.

20. Human insulin precursors of the general formula

$$B(1\text{-}29)\text{-}X_n\text{-}Y\text{-}A(1\text{-}21) \qquad II$$

wherein X_n is a peptide chain with n amino acid residues, n = 0 - 33, Y is Lys or Arg, B(1-29) is a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) is the A chain of human insulin, with the proviso that the peptide chain $-X_n-Y-$ does not contain two adjacent, basic amino acid residues.

21. A human insulin precursor according to claim 20 having the formula

B(1-29)-Ala-Ala-Lys-A(1-21).

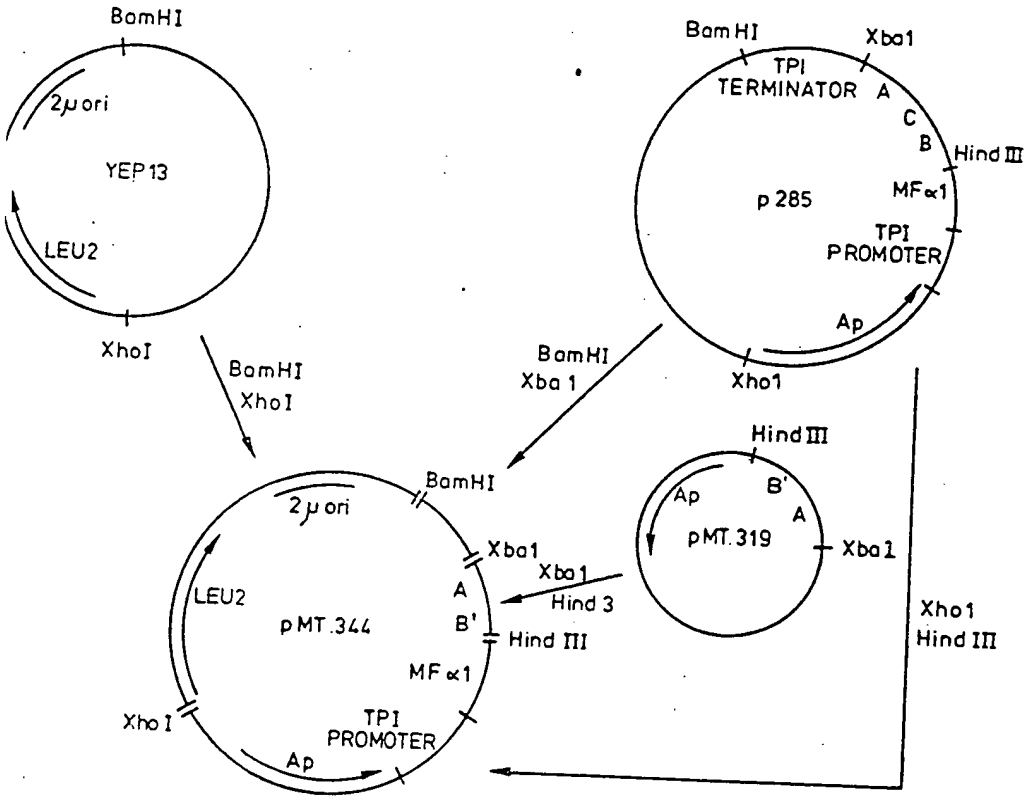
22. A human insulin precursor according to claim 21 having the formula

B(1-29)-Ser-Lys-A(1-21).

A B S T R A C T

Human insulin precursors containing the peptide chain B(1-29)-A(1-21) of human insulin and derivatives thereof with a bridging chain connecting the carboxyl terminus of the B(1-29)-chain with the amino terminus of the A(1-21)- chain are prepared by culturing a yeast host transformed with a replicable expression vehicle capable of expressing a DNA-sequence encoding the insulin precursor. The bridging chain is preferably relatively short and contains preferably from 2 to 8 amino acid residues. The bridging chain must not contain two adjacent basic amino acid residues (Lys or Arg) and has one Lys or Arg connected to the amino terminus of the A(1-21)- chain. Human insulin is prepared from the insulin precursors by in vitro conversion.

FIG. 1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C
B
Hind III
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PROMOTER
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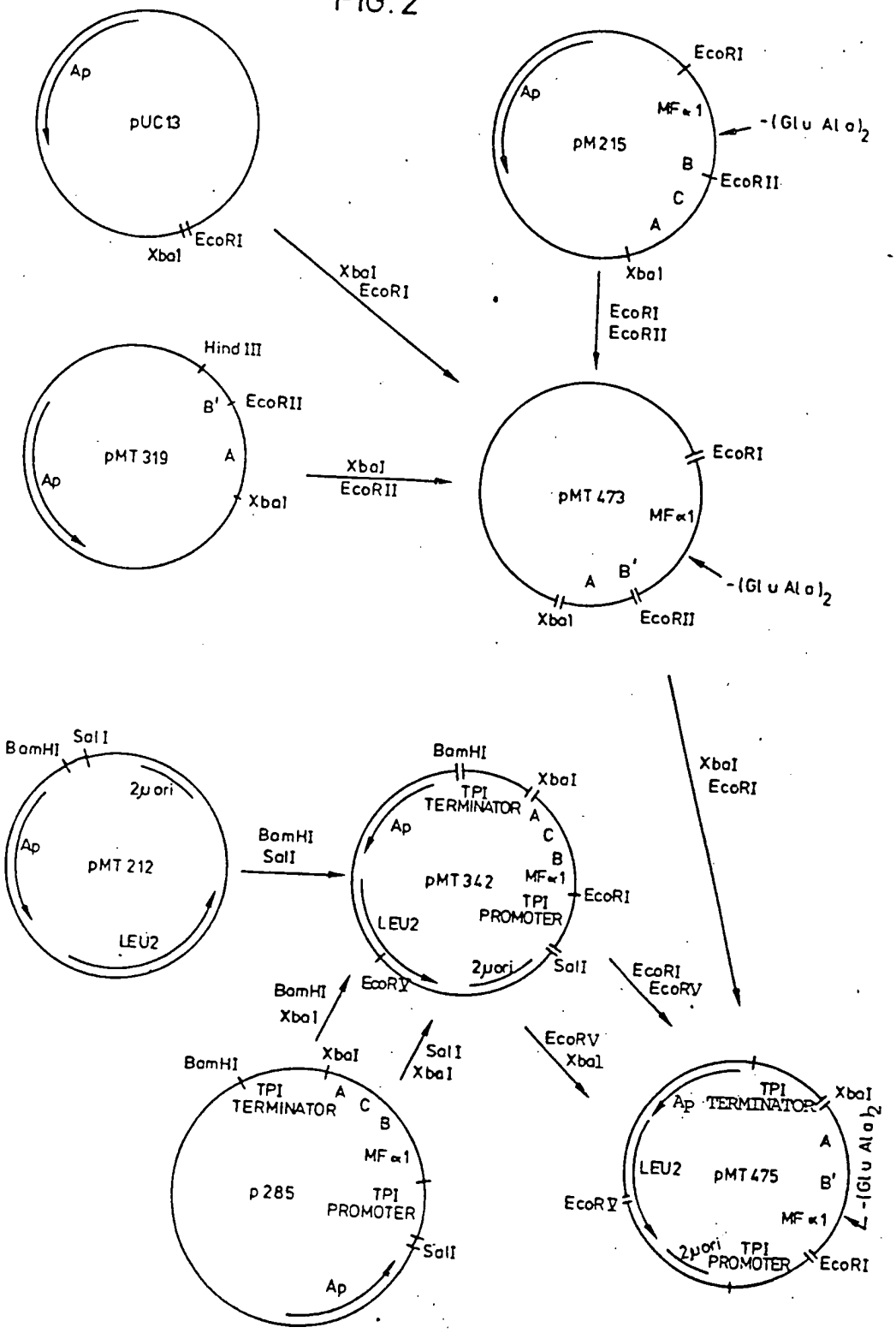
FIG. 2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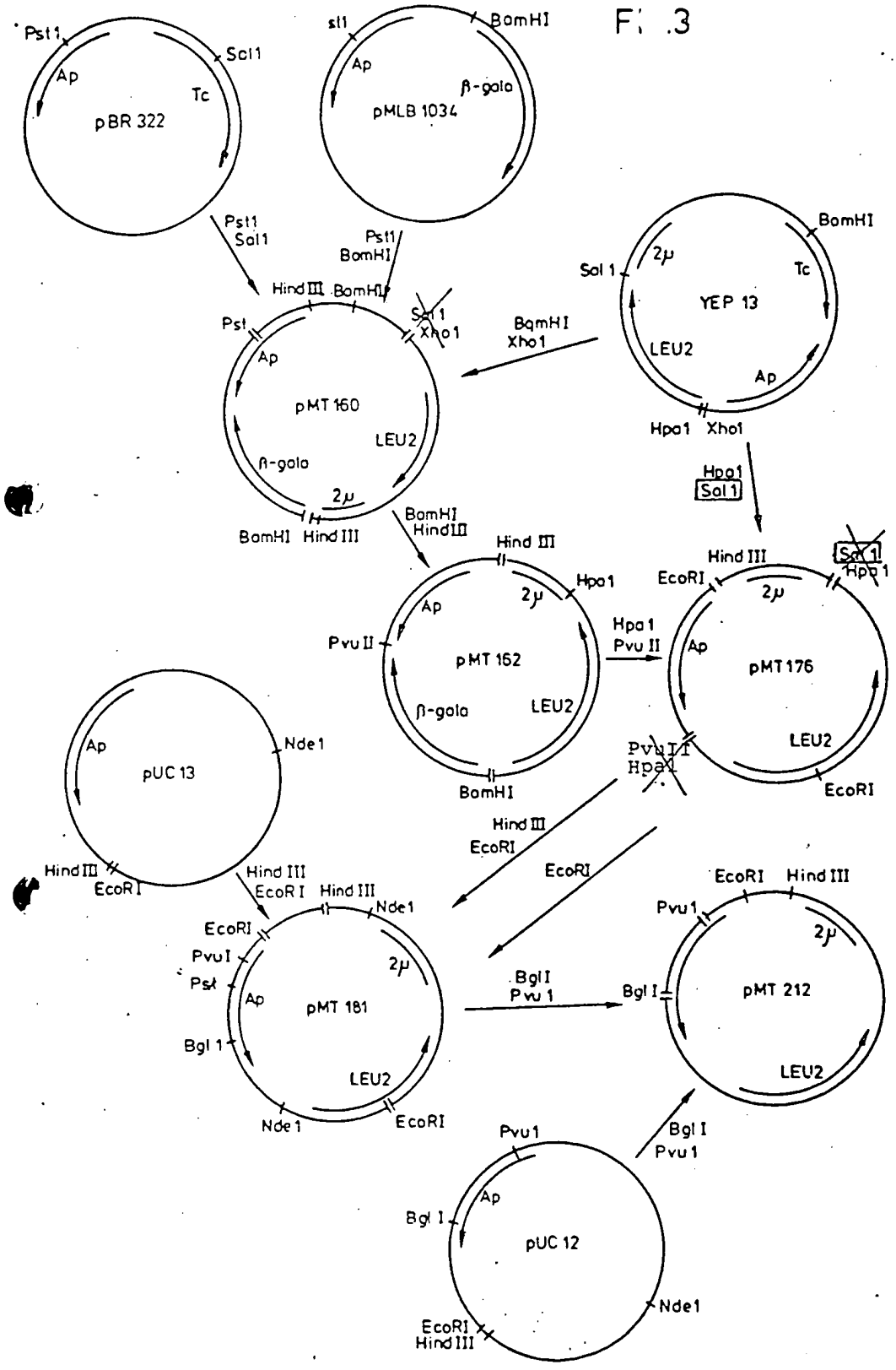
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Sal 1
Hpa 1
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2μ
Ap
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Pvu1
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Bgl 1
pUC 12
Nde1
EcoRI
Hind III

2/

#3

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

JAN MARKUSSEN et al.

Serial No. 739,123 Art Unit: 128

Filed: May 29, 1985

For: **DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INULIN**

LETTER

Honorable Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

Herewith are substitute formal drawings for Figures 3 and 8 as requested.

Respectfully submitted,

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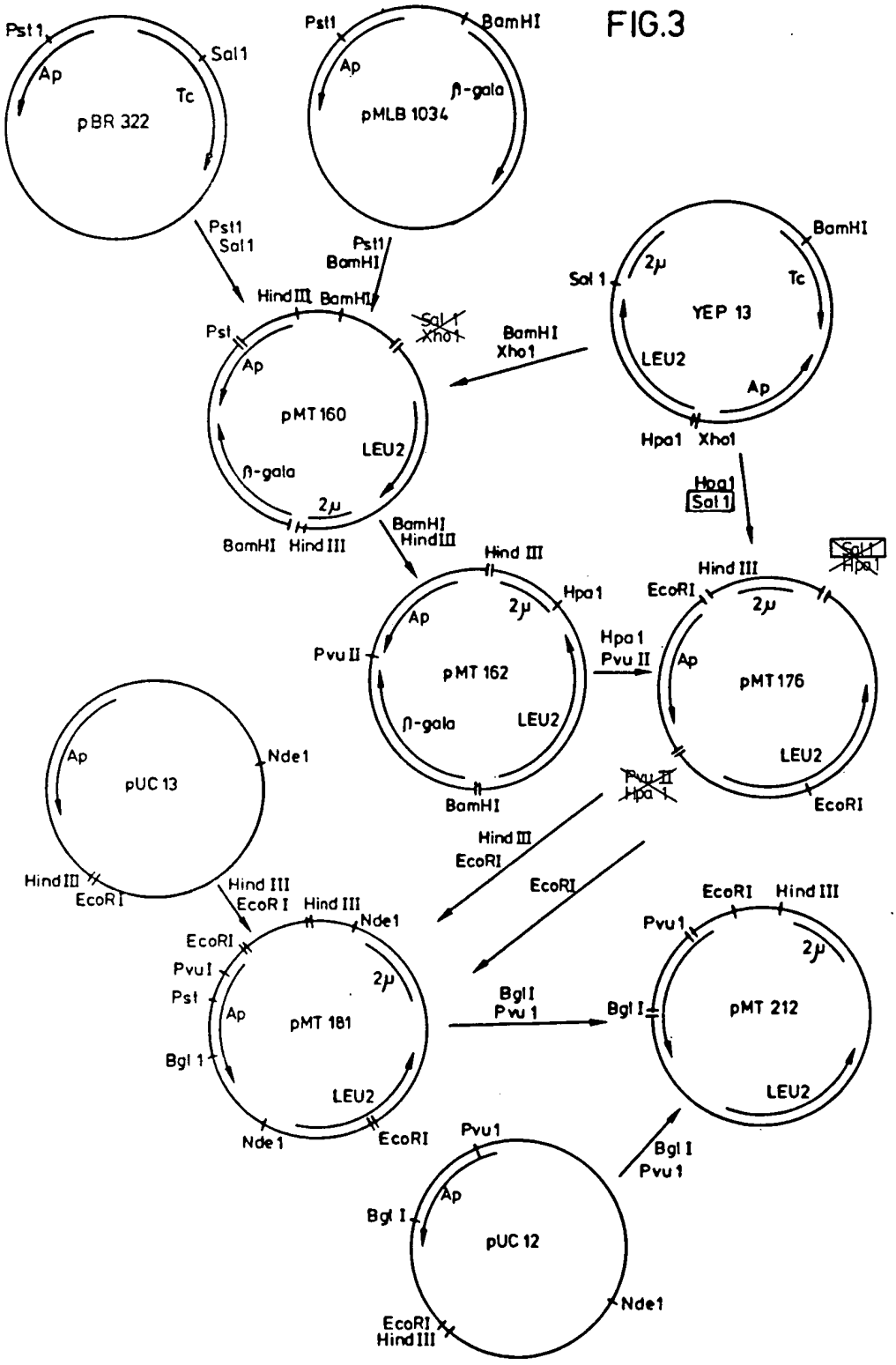
Dated: March 5, 1986
MF:js

FIG.3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2μ
BamHI
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Hpa1
Xho1
Pst
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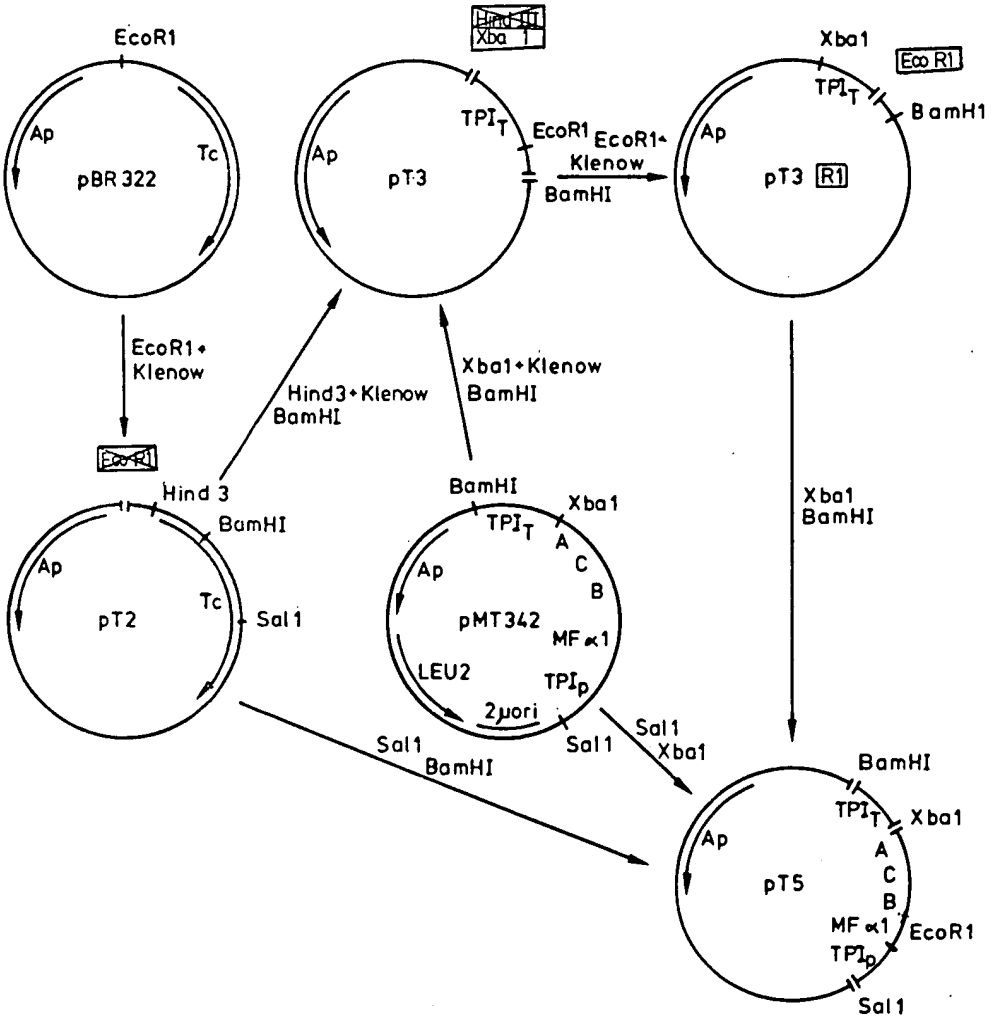
FIG. 8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A
C
B
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EcoR1
TPI$_p$
Sal1

12C

#4
cm
5/16/87

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

JAN MARKUSSEN, et al

Serial No.: 739,123 Art Unit: ~~128~~ 127

Filed: May 29, 1985 Examiner: Marschall

For: DNA-SEQUENCE ENCONDING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PROCURSORS AND HUMAN INSULIN

ASSOCIATE POWER OF ATTORNEY AND CHANGE OF ADDRESS

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

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If not previously appointed, the undersigned principal attorney of record hereby appoints as associate attorneys/agents with full power to prosecute this application and conduct all business at the Patent and Trademark Office the following attorneys of the above firm: Harold C. Wegner, Reg. No. 25,258; Barry E. Bretschneider, Reg. No. 28,055; Helmuth A. Wegner, Reg.

No. 17,033; Herbert I. Cantor, Reg. No. 24,392; Ronald R. Snider, Reg. No. 24,962; Douglas P. Mueller, Reg. No. 30,300; Elizabeth Lassen, Reg. No. 31,845; Evelyn K. Merker, Reg. No. 19,605; William E. Player, Reg. No. 31,409; Mel R. Quintos, Reg. No. 31,898; J. Derek Vandenburgh, Reg. No. 32,179; Kenneth E. Jaconetty, Reg. No. 32,508 and Lynn V. Kent, Reg. No. 31,904.

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Date: May 5, 1987

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/739,128	05/29/85	HANNOSSEN	[illegible]

FIDELMAN AND WOLFFE
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WASHINGTON, DC 20036-8218

EXAMINER	
CARSON, [illegible]	
ART UNIT 185	PAPER NUMBER 5

DATE MAILED: 04/25/88

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

☑ This application has been examined ☑ Responsive to communication filed on 5/6/87 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), ___ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☑ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☑ Claims 1-32 are pending in the application.

   Of the above, claims _____ are withdrawn from consideration.

2. ☐ Claims _____ have been cancelled.

3. ☐ Claims _____ are allowed.

4. ☑ Claims 1-30 are rejected.

5. ☐ Claims _____ are objected to.

6. ☐ Claims _____ are subject to restriction or election requirement.

7. ☐ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.

8. ☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.

9. ☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).

10. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

12. ☑ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☑ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.

13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

14. ☐ Other

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification is objected to under 35 U.S.C. 112, first paragraph, as failing to provide an enabling disclosure. Although it appears that applicant has deposited the biological material necessary to practice the disclosed invention, it does not appear that applicant has complied with MPEP 608.01(p)(C).

A deposit of biological material is generally required when all of the starting materials are not available to the public and when applicant has not presented the complete nucleotide sequence of the DNA essential for practicing the claimed invention. A deposit must satisfy the availability and maintenance requirements of MPEP 608.01(p)(C). In regard to permanence, a period of 30 years after the deposit, 5 years after the last request or the enforceable life of the patent, whichever is longer has been found to be adequate. The deposits should be available to the

Commissioner during pendency and all restraints upon availability should be irrevocably removed upon issuance of a patent. Assurance of compliance may be in the form of an averment under oath or declaration, and submission of a photocopy of the record of deposition received by applicants from the depository. Please note that the deposited plasmid strain should be accurately cross-referenced between the oath/deposition and record of deposit.

Claims 1-32 are rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the above objection to the specification.

Claims 19-23 and 25-27 are rejected under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited 1-18, 24 and 28-32. See MPEP 706.03(n) and 706.03(z).

The disclosure is not enabling for the production of "mutants or variants thereof" as claimed. Applicants do not disclose any type of mutants or variants of the exemplified strains nor do they disclose techniques by which these mutants or variants may be obtained. Absent, this information it is deemed that it would require undue experimentation by one of ordinary skill in the art to produce the claimed invention.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.
>
> Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Claims 1-6, 12, 18, 20, 22, 24, 29-32 are rejected under 35 U.S.C. 103 as being unpatentable over Brake et al or Kingsman et al in view of Markussen and Narang et al.

Brake et al teaches the production of proinsulin in yeast by joining the gene encoding proinsulin to yeast secretion and processing signals present on a yeast vector with subsequent replication in a yeast host.

Kingsman et al teach yeast vectors capable of expressing heterologous proteins in yeast cells.

Markussen teaches the B(1-29) A(1-21) insulin moiety and production of human insulin from this linear peptide.

Markussen further teaches that an intermediate compound in the conversion of the B(1-29) ACl-21 moiety to human insulin, has additional peptides at the carboxyl terminus of the B(1-29) chain and at the amino terminus of the A(1-21) chain with a basic amino acid residue immediately adjacent the first amino acid residue of the A chain.

Narang et al. teaches the production of insulin precursons through cloning and expression of the DNA sequences encoding the A and B chains along with a modification of the C peptide. Narang et al teach that modification of the C peptide gives a higher yield of biologically active insulin. Narang et al futher teach the sensitivity of, proinsulin to peptidase cleavage of dibasic sequences within the additional peptide sequence found between the carboxy terminus of the B chain and the amino terminus of the A chain.

In the absence of unexpected results it would be obvious to one of ordinary skill in the art to clone a contiguous DNA sequence encoding the intermediate compound disclosed by Markussin with the modifications

suggested by Narang et al to the vector described by Brake et al or Kingsman et al, in order to obtain undegraded insulin precursor molecules that require minimal additional processing to produce nature insulin.

Claims 7-11, 13-17, 19, 21, 23, 25 and 27-28 are rejected under 35 U.S.C. 103 as being unpatentable over Brake et al or Kingsman et al in view of Markussen and Narang et al as applied to claims 1-6, 12, 18, 20, 22, 24, 26 and 29-32 above, and further in view of inventors admitted state of the art.

It is deemed that it would be obvious to one of ordinary skill in the art to utilize known promotors and secretion elements such as those described on pages 11 and 12 of the specification to produce the vectors and transformed hosts claimed.

The Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 185.

Any inquiry concerning this communication should be directed to Patricia A. Carson, PhD at telephone number 703-557-1095.

Carson:bjk
4/18/88

THOMAS G. WISEMAN
SUPERVISORY PATENT EXAMINER
ART UNIT 127

TO SEPARATE TOP AND BOTTOM EDGES, SNAP-APART ANT CARD CARBON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
739123	185	8

APPLICANT(S): Hartussen et al

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
(R)	L	0056445	7/4/82	EP	Ganadol	C12N	15/00		
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R Greene et al Methods in Enzymology Vol. XLVII 1977 p170-172

S

T

U

EXAMINER	DATE	
Carlson	12/30/88	Carlson

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

$390.00-117 M.P.

180 641
4/25/88

185
11/1/88

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

JAN MARKUSSEN, ET AL Art Unit: 185

Serial No: 739,123 Examiner: P. Carson

Filed: May 29, 1985

For: **DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN**

LETTER ACCOMPANYING AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Enclosed herewith is an AMENDMENT for filing as of this date; kindly also make of record the following:

FEES FOR AMENDED CLAIMS

Excess independent claims at $34 each -	$ -
Excess total claims at $12 each -	$ -
First multiply dependent claim at $110 extra -	$ -

EXTENSION OF TIME PETITION

If this paper is filed outside the regular shortened period for response, applicant(s) petition(s) for the minimum extension of time needed to effect timely filing of the instant paper, calculated as being for a total of 3 months, and the fee being $ 390.00

[x] TOTAL FEE: Our check is included for: $ 390.00

070 10/26/88 739123 1 117 390.00 CK

[x] Applicant(s) generally authorize(s) payment of any required fee for the filing of this paper (even if different from any calculation above) to our Deposit Account 23-0783 under our general authorization under 37 CFR 1.17.

Respectfully submitted,

Morris Fidelman

Morris Fidelman
Reg. No. 17,126

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Atty. Docket: P-6315-20934
Date: October 24, 1988
MF:as

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

JAN MARKUSSEN, ET AL

Serial No: 739,123 Art Unit: 185

Filed: May 29, 1985 Examiner: Carson

For: DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

AMENDMENT

Honorable Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

In response to the outstanding Office Action dated April 25, 1988, please amend the above-referenced application as follows:

IN THE SPECIFICATION

At page 8, lines 19, 20, change "Danish Patent Application 574/80" to -- U.S. Patent 4,343,898 --.

IN THE CLAIMS

Claims 19, 21, 23,25, 27, line 3, delete "variant or mutant".

REMARKS

Reconsideration of the above identified application is requested in light of the forgoing amendment and the following remarks.

The specification was objected to and Claims 1-32 were rejected under 35 USC 112, first paragraph for a purported failure to provide an enabling disclosure and to comply with MPEP. 608,01(P)(C). The objection and rejection are in error.

All starting material used in the present invention are known or can be made as described in great detail in the present specification. The structure of insulin and the insulin gene were both well known before the filing date of this application. Isolation of human proinsulin C-DNA was made by known methods and described on pages 9-10. Also methods to mutate gene, e.g, by site specific mutagenesis were well known on the filing date (see page 10). The mutation is furthermore described in detail on pages 14-18.

Methods for synthesis of oligonucleotides were also well known in the art at the filing date of the patent application and assembly of the oligonucleotides to DNA-sequences were also well known and are described in the specification. The starting plasmids used are described in the literature and some are available from commercial sources. In general, the

particular starting plasmids were selected on the basis of convenience, as they were available in the inventors laboratory at the time the invention was made. The ordinarily skilled person in the art of molecular biology would recognize that equivalent materials can be used. Based on the present specification the expert in the art would know how to make a DNA-sequence encoding the insulin precursors in question knowing their protein sequence which is given by formula I. He would then know how to combine such a sequence with suitable yeast promoters and leaders and how to transform yeast.

To summarize, the recombinant DNA-technology used was well known on the filing date. It was furthermore well known to transform yeast and it is correct that some of the plasmids described by Kingsman might be usable to express the present insulin precursors if the gene for such products were inserted in these plasmids.

With respect to the status of the various plasmids and microorganisms NOVO has deposited the yeast strains MT 350, MT 371, MT 519, MT 620 and MT 643 (see Table 1) containing the plasmid mentioned in the same Table. The strains (and plasmids) were deposited for patenting purposes according to the Budapest Treaty. This means that they are available by request by third party and will be held available for 30

years by the depository organization. Hereto attached is a copy of Applicants, record of deposition received by DSM. No oath or declaration should be necessary. However, it is Applicants' position that the deposited plasmids are only exemplary of preferred embodiments of the invention, but they are not required to perform the invention. The deposited plasmids and strains as well as equivalents thereto may be constructed by one of ordinary skill in the art using readily available starting materials and following the guidance provided in our specification.

Therefore, the rejection of Claims 1-32 and the objection to the specification under 35 USC 112 should be withdrawn.

Reconsideration of the rejection of Claims 19-23, 25-27 under 35 USC 112 is requested in light of the foregoing Amendment. This rejection was based upon recitation of "variant or mutant" in the claims 19, 21, 23, 25, 27. By the above amendment, the term "variant or mutant" has been deleted.

The claims herein, namely 1-32 have been rejected under 35 USC 103 as unpatentable over Brake et al or Kingsman in view of Markussen and Narang et al. This rejection is inappropriate and should be withdrawn. Parenthetically, it

is noted that the Denmark version of Markussen 4,343,898 was referenced at page 8. The U.S. patent is now referenced.

A unitary theme of patentable invention extends through all the claims, as may be seen from the independent Claims 1, 6, 12, 18, 29, 30. They all relate to the various aspects of producing the B(1-29)-(X_n-Y)m-A(1-21) proinsulin analogs, namely, the proinsulin analogs themselves (Claim 30), the cultivation process productive of the analogs (Claim 18), the method for preparing a transformant yeast, then cultivating same, etc. (Claim 29), the transformed yeast strain (Claim 12), the expression vehicle (Claim 6) and the DNA-sequence (Claim 1).

To repeat, the subject matter common to all claims herein is the B(1-29)-(X_n-Y)$_m$-A(1-21) group of human insulin precursors that is not taught or suggested by Kingsman et al 4,615,974, Brake et al EPO 0 121,884 and Narang et al EPO 0 068,701. As the Examiner has recognized: Brake et al teaches production of proinsulin from yeast cells (pre-proinsulin really); Kingsman et al relates to plasmid vectors for yeast transformation; and, Narang teaches curtailing and/or lengthening the C-chain in human proinsulin.

To a great extent Brake et al teach how human proinsulin may be prepared by in yeast through recombinant DNA techniques. Narang et al teach how to make proinsulin in E.

coli and that the yield of human miniproinsulin may vary depending upon the peptide sequence of the proinsulin precursor. Thus, their teachings extend beyond the exemplary disclosure into an invitation to invent, which is to say, they leave future workers in the art the task of discovering particular superior insulin precursors. Since no certainty exists that truly superior insulin precursors will ever be discovered or even that they exist, the law under 35 USC 103 has long since ruled in such circumstances that their discovery would constitute an act of invention. The discovered advantageous process and/or products are patentable. The Examiner is not free to urge that any particular novel advantageous human insulin precursor is made obvious (under 35 USC 103) by Narang et al, Brake et al, Kingsman et al, either individually or in any combination thereof.

The Examiner has probably appreciated the state of the law under 35 USC 103 which applies to the "discovery" situation as witness the proviso "In the absence of unexpected results" offered (at page 5 bottom) to the rejection under 35 USC 103. Unexpected advantageous results exist.

As is described in Applicants' specification the insulin precursors of this invention are truly superior.

Important to the rejections under 35 USC 103 is the import <u>vis a vis</u> Applicants' claims that should be accorded to Markussen 4,343,898 (the same Jan Markussen being an inventor herein). The Examiner has interpreted 4,343,898 as relating directly to recombinant DNA technology.

Markussen 4,343,898 describes the conversion of the insulin polypeptide moiety.

```
- (1--A--21)
      ||
  (1--B--29) -
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into h-In-Thr^{B30} esters. One human insulin precursor so converted is

```
(1--A--21)
    ||
(1--B--29) - Ala,
```

namely porcine insulin.

Other insulin-like polypeptides which may be converted are human proinsulin, porcine diarginine insulin, etc. (see Col. 6, lines 5-36).

Thus, an aspect of the present invention is that the teachings in Markussen 4,343,898 are directly applicable to the recombinant human insulin precursors capable of being elaborated by transformants (e.g., transformed yeast). The Examiner seems to have treated this aspect of the invention as being part of the prior art.

Another aspect of the invention resides in Applicants' discovery of particular human insulin precursors that are

well elaborated by transformant yeast cells. Such insulin precursors do not include the most characteristic feature of the C-chain polypeptide in human proinsulin. The C-chain polypeptide of human proinsulin, (see page 3 of 0068701), contains Arg-Arg and Lys-Arg as the (proinsulin) amino acid residues 31, 32 and 64, 65 respectively.

Thus, the present invention advances over the prior art Narang et al, Brake et al and Kingsman et al teachings. Applicants prepare a novel group of human insulin precursors that may be elaborated in high yield by appropriately transformed yeast cells. Vis a vis the recombinant DNA prior art, Applicants' human insulin precursors are unobviously advantageous by reason of good cultivation yield. Yield data is given at Table 1 herein (at page 13). Enclosed is an article by Lars Thim et al. from PNAS 83 (1986), 6766-6770 where the low yields of proinsulin and miniproinsulin appear from Table 1 (compounds 1-6). These results can be directly compared to the results in Table 1 in the present text as the tests were conducted under identical conditions.

Applicants' human insulin precursors are unobviously advantageous, also, in that they may be converted in high yield through transpeptidization of the insulin precursors into Thr B30 esters of human insulin, and then into human insulin by the process of Markussen 4,343,898.

This capability to fit into the established technology of Markussen 4,343,898 does not (retrospectively) make the novel proinsulin polypeptide analogs of this invention obvious. Indeed, such capability offers reason why the novel proinsulin analogs are unobvious and patentable under 35 USC 103.

Over and above patentability of the independent Claims 1, 6, 12, 18, 29, 30 under 35 USC 103 is separate patentability for the more limited dependent claims. The dependent claims, one way or another, relate to preferred modes of the invention comprising a short connecting C-chain peptide. Preferred embodiments include B(1-29)-A(1-21), a polypeptide which in and of itself is known (see page 2, lines 20-31 of Applicants' specification), and the novel polypeptides B(1-29)-Ala-Ala-Lys-A(1-21), B(1-29)-Ser-Lys-A(1-21); and, more generically, a C-chain of 1-8 residues (see Claim 3) without two adjacent basic amino acid residues. The prior art fails to teach or suggest the claimed human proinsulin precursors. To repeat, they are advantageous for being elaborated in high yield by yeast transformants and for fitting so well into the chemical conversion sequence described in Markussen 4,343,898.

Narang et al 0068701 offers several prophetic thought at page 16, lines 3-12, speculating in particular that changing

the length of the human proinsulin C-chain generated in transformed E. coli might be advantageous. The Narang et al prophetic thoughts constitute a classic invitation to invent particular shortened C-chain insulin precursors. Under the law of 35 USC 103 which applies to discoveries in the biologic arts patentability exists in any and all advantageous shortened C-chain insulin precursors generated by a worker in the art who carried out the Narang et al suggestion.

The conceptual approach of this invention does not directly carry out the Narang et al suggestion. Instead Applicants are advancing the art over the Narang et al suggestion (a distinct, if subtle difference). Since Applicants already know that a wide variety of insulin-like compounds of Formula 1 in 4,343,898 convert into human insulin, see Col. 5, lines 36-60, and the lengthy list in Column 6 of 4,343,898, which list includes human proinsulin, they looked to discovering human insulin precursors that can be generated by a transformed yeast in high yield. One such polypeptide turns out to have no C-chain at all, being B(1-29)-A(1-21). Other polypeptides have a connecting chain, but Applicants' connecting chain (denominated herein as $-(X_n-Y-)_m$) has Lys or Arg for Y but does not contain two

adjacent basic amino acid residues. The C-chain in human proinsulin contains 31-Arg-32-Arg and 64-Lys-65 Arg.

Thus, although the connecting chain in Applicants polypeptides might be considered as shortened C-chain proinsulins to confirm to the prophetic statements made in Narang et al, they confirm Narang et al in a completely unexpected fashion, i.e., through deletion of the paired basic amino acid residues that Narang et al expects will remain the insulin precursors.

The rejections under 35 USC 103 are inappropriate and should be withdrawn.

A prompt reexamination and passage of this application to issue is solicited.

Respectfully submitted,

Morris Fidelman
Reg. No. 17,126

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(202) 833-8801

Atty. Dkt. P-6315-20934
Date: October 24, 1988

MF:as
Enclosures

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark ffice

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/739,123	05/29/85	MARKUSSEN J	104MF2196

IDELMAN AND WOLFFE
P.O.BOX 18216
WASHINGTON, DC 20036-8216

EXAMINER	
CARSON, P	
ART UNIT	PAPER NUMBER
165	8

DATE MAILED: 01/17/89

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

☑ This application has been examined ☑ Responsive to communication filed on 10/24/88 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), — days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☑ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
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Part II SUMMARY OF ACTION

1. ☑ Claims 1-32 are pending in the application.

   Of the above, claims _____ are withdrawn from consideration.

2. ☐ Claims _____ have been cancelled.

3. ☐ Claims _____ are allowed.

4. ☑ Claims 1-32 are rejected.

5. ☐ Claims _____ are objected to.

6. ☐ Claims _____ are subject to restriction or election requirement.

7. ☐ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.

8. ☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.

9. ☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).

10. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____. has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

12. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.

13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

14. ☐ Other

Claims 1-3, 12-15, 18-19, 21, 23, 25, 27 and 29-30 are rejected under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited insulin precursors specifically enabled by the as filed specification. See MPEP 706.03(n) and 706.03(z).

The specification discloses only the sequence and production of insulin precursons with no C chain or 2 specific C chains of Lys and arg. A great deal of unpredictability exists when altering DNA sequences for expression of a precursor molecule for production of a particular protein. It cannot be determined from the three functional precursors disclosed what additional C chain sequences of the same general formula would be useful in the claimed invention. In the absence of additional functional examples the scope of the claims is deemed to exceed the enabling specification.

Claims 1, 6, 12, 18, 29 and 30 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

These claims are vague and indefinite in the recitation of "naturally occurring amino acids". It is not clear what amino acids are being ~~required~~ referred to.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless-

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3 and 30 are rejected under 35 U.S.C. 102(b) as being anticipated by Goeddel et al.

Goeddel discloses insulin precusors which could be described by the general formula of claims 1-3. (See pages 8-9).

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.
>
> Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and

> the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Claims 6, 12-15, 18-19, 21, 23, 25, 27 and 29 are rejected under 35 U.S.C. 103 as being unpatentable over Goeddel et al in view of Brake.

Goeddel et al teach insulin precursors described by the general formula B(1-29) $(X_n\text{-}Y)_m$-A(1-21). He further teaches the expression of the cloned DNA sequences in bacterial host cells. Brake et al teach the advantages of producing cloned insulin precursors in yeast eg. efficient production and processing. In the absence of unexpected results it would be obvious to one of ordinary skill in the art to express the precursors taught by Goeddel et al in yeast for the advantages taught by Brake et al.

Claims 31-32 are rejected under 35 U.S.C. 103 as being unpatentable over Markussen et al in view of Greene et al.

Markussen et al teaches a transpeptidation reaction in the presence of trypsin for the conversion of insulin precursors to human insulin. Greene teaches the specificity of trypsin. In the absence of unexpected results it would be obvious to one produce precursors which contain amino acid residues suspeptible to

trypsin cleavage which can be converted to human insulin by the transpeptidation reaction taught by Markussen et al.

Claims 4-5, 7-11, 16-17, 20, 22, 24 and 28 are free of the prior art.

Any inquiry concerning this communication should be directed to Examiner Patricia Carson at telephone number 703-557-3871.

P. Carson/dal

1/9/89

THOMAS G. WISEMAN
SUPERVISORY PATENT EXAMINER
ART UNIT 185

TO SEPARATE, HOLD TOP AND BOTTOM EDGES, SNAP-APART AND DISCARD CARBON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER	
NOTICE OF REFERENCES CITED	739,123	127		
	APPLICANT(S) Markussen et al			

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A	4343898	8/10/82	Markussen	435	70	
	B	4615974	10/7/86	Kingsman et al	435	172.3	
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
X	L	0068701	1/5/83	EPO	Narang	C12 [redacted]	N15/00 [redacted]		
	M	0121884	10/17/84	EPO	Brake et al	C12	N15/00		
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R	
S	
T	
U	

EXAMINER	DATE	
Carson	9/21/89	Carson

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.05 (a).)

#9
Rpls 7-18-89

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

JAN MARKUSSEN, ET AL

Serial No: 739,123 Art Unit: 185

Filed: May 29, 1985 Examiner: Carson

For: **DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN**

PRIOR ART STATEMENT

9

Honorable Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

To complete the record herein, Applicants herewith submit copies of the prior art cited by the Examiners responsible for the various foreign counterparts to the above-referenced Application. Duplication has been avoided.

The art is believed to be only of general interest.

Respectfully submitted,

Morris Fidelman
Reg. No. 17,126

FIDELMAN & WOLFFE
P.O. Box 18218
Washington, D.C. 20036
(202) 833-8801

Atty. Dkt. P-6315-20934
Date: July 12, 1989

MF:as
Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

JAN MARKUSSEN, ET AL

Serial No: 739,123 Art Unit: 185

Filed: May 29, 1985 Examiner: Carson

For: DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

LETTER ACCOMPANYING AMENDMENT

Honorable Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

Enclosed herewith is an AMENDMENT for filing as of this date; kindly also make of record the following:

FEES FOR AMENDED CLAIMS

Excess independent claims at $36 each -	$ -
Excess total claims at $12 each -	$ -
First multiply dependent claim at $120 extra -	$ -

EXTENSION OF TIME PETITION

If this paper is filed outside the regular shortened period for response, applicant(s) petition(s) for the minimum extension of time needed to effect timely filing of the instant paper, calculated as being for a total of 3 months, and the fee being $430.00

[X] TOTAL FEE: Our check is included for: $430.00

[X] Applicant(s) generally authorize(s) payment of any required fee for the filing of this paper (even if different from any calculation above) to our Deposit Account 23-0783 under our general authorization under 37 CFR 1.17.

Respectfully submitted,

Morris Fidelman
Reg. No. 17,126

FIDELMAN & WOLFFE
P.O. Box 18218
Washington, D.C. 20036-8218
(202) 833-8801

Atty. Docket: P-6315-20934
Date: July 12, 1989

MF:as
Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

JAN MARKUSSEN, ET AL

Serial No: 739,123 Art Unit: 185

Filed: May 29, 1985 Examiner: Carson

For: DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

AMENDMENT

Honorable Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

In response to the outstanding Office Action dated January 17, 1989, please amend the above-referenced application as follows:

IN THE CLAIMS

Claims 19, 21, 23, 25, 27, line 3, please delete "or a thereof", so that this amendment and the prior amendment have deleted the phrase "or a variant or mutant thereof".

Claim 1, line 4, Claims 6 and 12, line 5, please delete "naturally occurring".

Claim 18, line 4, Claim 29, line 7, and Claim 30, line 3, change "naturally occurring" to read -- expressible --.

REMARKS

Reconsideration of the rejection in the above-identified Application is respectfully requested in light of the foregoing amendment and the following remarks.

In reviewing the file of this Application incident to preparation of the present amendment, the undersigned noticed that his previous amendment had completely garbled the intended deletion of the mutant or variant phase (from the claims). The above amendment offers correction so that the entire phase -- or a mutant or variant thereof -- is now cancelled.

In addition, the above amendment is intended to obviate the rejection of Claims 1, 6, 12, 18, 29 and 30 under 35 USC 112 as being indefinite. The Examiner objected to the phrase "naturally occurring amino acids". Applicants were of a mind to argue that point. The phrase "naturally occurring" is known in the art. It is not original with the Applicants. For example, the phrase naturally occurring can be found at page 5, line 30 of the EPO Publication 037255. However, after having decided to argue the point, Applicants realized that their explanation of, and argument about the term "naturally occurring" would involve identifying the naturally occurring amino acid residues as only the residues capable of being expressed by a transformed organism. From this line of thought evolved the above amendment to Claims 1, 6, 12, 18, 29 and 30. Why employ a synonym when the

exact terms of art -- expressed and expressible -- are supported in Applicants' specification?

Therefore, to resolve the Examiner's objection, the phrase "naturally occurring" has been deleted altogether from Claims 1, 6, and 12 by the above amendment as being redundant because a DNA-sequence is capable of encoding only for expressible amino acid residues.

Claims 18, 29 and 30 have been amended to employ the term "expressible" in substitution for "naturally occurring". The term "expressible" turns out to be quite apt since it ties in with the terms "replicable expression vehicle" and "expressing" already used in Claim 18, at line 13 and with comparable language in Claim 29, at lines 14 and 16.

Accordingly, Applicants submit that the rejection under 35 USC 112, second paragraph of Claims 1, 6, 12, 18, 29, and 30 is inappropriate to the claims as amended above and should be withdrawn.

Claims 1-3, 12-15, 18, 19, 21, 23, 25, 27, and 29-30 were rejected under 35 USC 112, first paragraph for lack of enablement. Reconsideration and withdrawal of this rejection is requested.

In explanation of the rejection the Examiner cited MPEP 706.03(Z), a section directed to the rejection of claims for undue breadth. Without evidentiary support the Examiner posed a statement as follows.

> "It cannot be determined from the three functional precursors disclosed what additional C-chain sequences of the same general formula would be useful in the claim invention."

The Examiner is in error on the law and on the facts. As to the law Applicants note that they have presented examples of three functional precursors in support of their general formula. Thus, Applicants' statements in the specification about the formula and the support for such statements by exemplary material to several species that fit the formula amount to a prima facie case for the entire formula that they have alleged. Accordingly, the further comment by the Examiner "in the absence of additional functional examples, etc." is not justified in law or in practice. A general knowledge that unpredictable results follow from altering DNA sequences does not satisfy the Examiner's burden of proof. The MPEP 706.03(2) describes the standard for requiring exemplification by more than one species, i.e., unpredictability. Given unpredictability, one specific example is not enough. The general knowledge that unpredictability established proved (prima facie) that Applicants could not rely on an exemplary disclosure of a single species. By posing examples to three functional (preferred) insulin precursors Applicants sought to dispel concern over unpredictability. The evidence of three functional examples rebuts general unpredictability. Now Applicants have submitted a prima facie case for their formula. They have established that a degree of

predictability exists. Now, the Examiner must provide more evidence in support of the rejection. Since none has been provided, Applicants' position must prevail.

The Examiner erred on the facts as well. To repeat, Applicants have posed only three functional examples but they have more evidence. Over and above their own experimental efforts to prove the genus of the formula, Applicants are entitled to rely on the prior art. In specific, Applicants may rely on prior art knowledge about variations in amino acid residues within naturally occurring proinsulins, one species to another species. The C-chain may vary in length from 30-35 amino acid residues and in individual residues as well.

In addition to their data, and data relating to naturally occurring proinsulins, Applicants are entitled to rely on the consensus in the art that the C-chain serves nothing more than a spacing function. The C-chain bridges the A and B chains and aligns them into the proper insulin configuration. At sometime thereafter the C-chain is excised. Therefore, the art would expect that considerable variation in C-chain length and amino acid residue content can be expected to have no detrimental effect, since only a spacing function is being served. Thus, in the area of this invention the art believes that little or no unpredictability exists. Accordingly, Applicants submit that the facts demonstrate that their formula does apply

without some unpredicted exception(s). In support of their above argument that the art is aware of many natural variations in C-chain length and amino acid residue content, and that the art believes that the C-chain serves only a spacing function, Applicants attach hereto photo copies of: Steiner, Diabetes 27 (Suppl. 1): 145-148, 1978; and, Chan et al, Diabetes Care, Vol. 4, No. 1, Jan-Feb. 1981, pp. 4-10.

The Examiner's rejection of the claims herein for reason of undue breadth is without basis in law or in fact and should be withdrawn.

Having said so much, Applicants note that they have made other insulin precursors and can present the data. How many functional precursors in total would satisfy the Examiner? So far Applicants have some eight additional precursors in hand.

The Claims 1, 2, 3, and 30 are rejected under 35 USC 102(b) as being anticipated by Goeddel et al (hereinafter Goeddel). This rejection is inappropriate and should be withdrawn because Goeddel does not anticipate Applicants' invention.

The insulin precursor formula set out in Applicants' claims names the B(1-29) chain, which is a shortened B-chain of human insulin. The B30 residue (Thr) present in human insulin is not there, but in their formula B-(1-29)-X_n-Y-A(1-21) Xn sequence of amino acid residues might be gin with Thr. Goeddel (at page 8) speaks of the A and B-chain

of human insulin and a C-chain, the latter being called a bridging chain and which according to Goeddel may comprise as few as two amino acid units. This requirement by Goeddel for the human insulin B-chain and some sort of bridging chain removes Claim 2 from the rejection under 35 USC 102 because Claim 2 calls for a DNA sequence which provides a protein with no bridging chain and the B(1-29) sequence.

Goeddel poses still another exclusionary comment, which comment reads "However, the end units thereof must be units which permit facile excision of the bridging chain from the A and B-chains of human insulin" (lines 25-27) and on page 9 starting at line 3 Goeddel expresses preference for Arg-Arg and Lys-Arg units adjacent to the COOH terminal of the B-chain and adjacent the terminal NH2 of the A-chain all of which line of reasoning allows for two Arg-Arg units between the B(1-30) and A (1-21) chains. Applicants' claims call for a peptide chain which does not contain two adjacent basic amino acid residues, i.e., may not have paired residues such as Arg-Arg or Lys-Arg. Applicants are not making a trivial point. The attached Steiner and Chan et al papers emphasize that presence of a pair of basic amino acid residues at each end of the C-chain has been conceived in species after species throughout considerable evolution.

Putting together the statements made by Goeddel at pages 8 and 9 some exclusionary requirements for the bridging chain have been set forth, namely, the end units of

the bridging chain must be residues which can be excised readily. Although the end units need not be the preferred Arg-Arg or Lys-Arg units Goeddel fails to name any acceptable but less preferred end units of the bridging chain. In light of the evolutionary preference for the paired basic residues, the reader is left only with conjecture as to the identity of less preferred end unit.

Thus, for Goeddel to say that the bridging chain need not terminate in a paired unit of Arg-Arg or Lys-Arg is not the same as a teaching or suggestion or an inherent disclosure of a bridging chain that terminates in a single Lys or Arg residue adjacent the COOH terminal of the B-chain.

Actually, the Examiner is unable to incorporate any practice of Applicants' invention within the metes and bounds of Goeddel. Within the scope of Goeddel, there are too many variations that are not Applicants' invention, the paired basic amino acid residues in particular and presence only of B(1-30). Applicants' B(1-29) invention must be suggested, or taught or inherently exemplified. Certainly, Applicants' invention is not taught nor is it somehow exemplified, all of which is to say that no rejection under 35 USC 102 can apply against Claims 1, 2, 3, and 30. This rejection should be withdrawn as inappropriate.

Claims 6, 12-15, 18, 19, 21, 23, 25, 27 and 29 were rejected under 35 USC 103 as being unpatentable over Goeddel

in view of Brake. This rejection is believed to be in error and should be withdrawn. Goeddel is very nearly as defective under the standards for patentability under 35 USC 103 as under the standards of 35 USC 102. There is no teaching or suggestion of a B(1-29) or of a single basic amino acid residue in the C-chain. Goeddel fails to suggest an insulin precursor wherein the bridging chain does not contain any paired basic amino acid residues, e.g., Arg-Arg. Goeddel expresses strong preference for paired basic amino acid residues adjacent the COOH terminal such as the Arg-Arg groups in human proinsulin, see page 9, lines 3-8. Only through absence of express exclusionary language in point was the Examiner enabled to identify Goeddel with a key limitation of Applicants' invention, i.e., the single basic amino acid residue.

It is not as if Applicants' invention is nearly encompassed within the Goeddel et al disclosure. Only a small overlap might exist at best. Applicants' formula calls for B(1-29)-$(X_n-Y)_m$ A(1-21). When m is 0 the formula as a whole is outside the scope of Goeddel. When m is 1 a strained interpretation of both Goeddel and Applicants' formula is necessary to generate the purported overlap, as witness that Applicants' B(1-29)-X_n can fall within the scope of the Goeddel disclosure only whenever the first residue of the X_n sequence is Thr. Goeddel must have the B(1-30) chain. Thus, when Applicants' (X_n-Y) bridging

chain is a peptide which commences with Thr an overlap is possible in theory provided that the last two amino acid residues in the sequence (i.e., those at the COOH terminal) also met the Goeddel requirements for the X_n and Y_1 residues.

To repeat, Applicants' C-chain may not terminate in paired basic amino acid residues. However, Goeddel does not teach or suggest (save through silence) any bridging chain containing any other terminal (X_n, Y) residues. Thus, no overlap actually exists but the Examiner has attempted to make one hypothetically possible. To do so Applicants' teachings must be fitted into the Procrustian bed of Goeddel, and it cannot.

Good reason exists for absence of even the narrow (hypothetical) overlap between the proinsulin analogs of Applicants and Goeddel urged by the Examiner. The overlap region must satisfy two different approaches to excision of the bridging chain. The technique employed by Goeddel employs the paired basic amino acid residues that allow excision of the bridging chain in facile fashion. Applicants employ the transpeptidation technology of Markussen 4,434,898 on the B(1-29)-X_n-Y-A)1-21) peptides.

The Examiner cited Brake as an ancillary reference for suggesting expression of the insulin precursors in yeast. The Examiner also commented about unexpected results. In the prior amendment Applicants discussed Brake, pointing out

that the data in the specification hereof and in the Thim et al paper demonstrate that Applicants' human insulin precursors are (unobviously) advantageous for their good cultivation yield. Applicants' prior remarks also pointed out that the transpeptidation technology of Markussen 4,434,898 will excise the bridging chain X_n-Y in high yield. Thus, for the insulin precursors of Applicants' invention the unobvious results attributed thereto are already demonstrated in the record. Does the Examiner insist on the formality of a Declaration? Accordingly, the rejection of Claims 6, 12-15, 18, 19, 21, 23, 25, 27, 29 over Goeddel in view of Brake is inappropriate and should be withdrawn.

Claims 31 and 32 are rejected under 35 USC 103 as being unpatentable over Markussen et al in view of Greene et al. This rejection may not be understood by Applicants.

Certainly, Markussen teaches the excision technology employed by Applicants herein. Markussen also teaches the specificity of trypsin. (Thus, Greene is not seen as a necessary reference.) The point of Claims 31, 32 is that the insulin precursors defined by these claims are unobvious for being expressible by the microorganism in high yield. The precursors are novel peptides which exhibit two advantages. One is, of course, yield. The other is that they can be transpeptidized well by the Markussen technology. Expression of insulin in high yield is of little use in real life without availability of a high yield

conversion procedure. The capability for a high yield conversion of insulin precursors having the formula

-(1-A-21)

(1-B-29)-

by the Markussen process does not make these particular insulin precursors obvious under 35 USC 103 over Markussen 4,343,898. Accordingly, unexpected results have been demonstrated for the compounds of Claims 30, 31, and 32. This rejection of Claims 31 and 32 under 35 USC 103 is inappropriate and should be withdrawn.

This Application has been filed in many countries. By and large the same prior art has been cited by all Examiners but not completely. Hereto attached is an Information Disclosure Statement listing the references cited in Denmark, the EPC, Australia and New Zealand (duplications omitted).

In conclusion, Applicants submit that the claims as above amended properly define their invention so as to patentably distinguish over the art.

Applicants acknowledge with appreciation that the Examiner indicated Claims 4, 5, 7-11, 16, 17, 20, 22, 24, and 28 to be free of the prior art.

A prompt reexamination and passage of this application to issue is solicited.

Respectfully submitted,

Morris Fidelman

Morris Fidelman
Reg. No. 17,126

FIDELMAN & WOLFFE
P.O. Box 18218
Washington, D.C. 20036
(202) 833-8801

Atty. Dkt. P-6315-20934
Date: July 12, 1989

MF:as
Enclosures

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
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Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Morris Fidelman (3) _____

(2) Pat Caesar (4) _____

Date of interview 9/21/89

Type: ☒ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☒ No. If yes, brief description: _____

Agreement ☒ was reached with respect to some or all of the claims in question. ☐ was not reached.

Claims discussed: 1-4

Identification of prior art discussed: none

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Agreement to amend claims 1-4 to recite "An isolated DNA sequence"

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

Patricia Caesar
Examiner's Signature

PTOL-413 (REV. 1-84)

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UNITED STATES DEPARTMENT OF COMMERCE
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Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	**PAPER NUMBER**
	17

DATE MAILED:

NOTICE OF ALLOWABILITY

PART I.

1. ☐ This communication is responsive to amendment filed 7/13/89.
2. ☑ All the claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice Of Allowance And Issue Fee Due or other appropriate communication will be sent in due course.
3. ☑ The allowed claims are 1-32.
4. ☐ The drawings filed on 11/5/86 are acceptable.
5. ☑ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has [✓] been received. [_] not been received. [_] been filed in parent application Serial No. _____, filed on _____.
6. ☑ Note the attached Examiner's Amendment.
7. ☑ Note the attached Examiner Interview Summary Record, PTOL-413.
8. ☑ Note the attached Examiner's Statement of Reasons for Allowance.
9. ☐ Note the attached NOTICE OF REFERENCES CITED, PTO-892.
10. ☑ Note the attached INFORMATION DISCLOSURE CITATION, PTO-1449.

PART II.

A SHORTENED STATUTORY PERIOD FOR RESPONSE to comply with the requirements noted below is set to EXPIRE THREE MONTHS FROM THE "DATE MAILED" indicated on this form. Failure to timely comply will result in the ABANDONMENT of this application. Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

1. ☐ Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL APPLICATION, PTO-152, which discloses that the oath or declaration is deficient. A SUBSTITUTE OATH OR DECLARATION IS REQUIRED.
2. ☐ APPLICANT MUST MAKE THE DRAWING CHANGES INDICATED BELOW IN THE MANNER SET FORTH ON THE REVERSE SIDE OF THIS PAPER.
   a. ☐ Drawing informalities are indicated on the NOTICE RE PATENT DRAWINGS, PTO-948, attached hereto or to Paper No. _____. CORRECTION IS REQUIRED.
   b. ☐ The proposed drawing correction filed on _____ has been approved by the examiner. CORRECTION IS REQUIRED.
   c. ☐ Approved drawing corrections are described by the examiner in the attached EXAMINER'S AMENDMENT. CORRECTION IS REQUIRED.
   d. ☐ Formal drawings are now REQUIRED.

Any response to this letter should include in the upper right hand corner, the following information from the NOTICE OF ALLOWANCE AND ISSUE FEE DUE: ISSUE BATCH NUMBER, DATE OF THE NOTICE OF ALLOWANCE, AND SERIAL NUMBER.

Attachments:

- ✓ Examiner's Amendment
- ✓ Examiner Interview Summary Record, PTOL-413
- ✓ Reasons for Allowance
- _ Notice of References Cited, PTO-892
- ✓ Information Disclosure Citation, PTO-1449
- _ Notice of Informal Application, PTO-152
- _ Notice re Patent Drawings, PTO-948
- _ Listing of Bonded Draftsmen
- _ Other

SUPERVISORY PATENT EXAMINER
ART UNIT 165

The following is an Examiner's Statement of Reasons for Allowance:

Claims 1-3,6,12-15,18-19,21,23,25 and 27 were previously rejected under 35 USC 112 first paragraph for lack of enablement and under 35 USC 102 and 103. In view of applicants arguments which set forth the prior art knowledge concerning the function of the C chain, natural variations in the length and amino acid content of the C chain and the prior art documents submitted to support these arguments, the rejection under 35 USC 112 is dropped

. Applicants have further pointed out several features which contribute to the superiority of the precursors of the instant invention which are not anticipated or suggested by Goeddel et al as applied to the claims under 35 USC 102 and 103. These features are the length of the B chain in the instant invention and the requirement for a bridging chain which does not contain two adjacent basic amino acid residues. In view of these distinctions the rejections under 35 USC 102 and 103 are dropped.

Any comments considered necessary by applicant must be submitted no later than the payment of the Issue Fee and, to avoid processing delays, should preferably accompany the Issue Fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

An inquiry concerning this communication should be directed to Pat Carson at telephone number (703) 557-3871

THOMAS G. WISEMAN
SUPERVISORY PATENT EXAMINER
ART UNIT 185

OMB No. 0651-0011 (12/31)

ATTY. DOCKET	Number
P-6315-. 24	S. 739,123

Patent Owner

Filing Date	GROUP
Filing 5/29/85	185

INFORMATION DISCLOSURE CITATION FOR PATENT

(Use several sheets if necessary)

MAIL ROOM JUL 12 1989 PAT. & TRADEMARK OFFICE

RECEIVED GROUP 180
JUL 14 1989

U.S. PATENT DOCUMENTS

PAGE 1 OF 2

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
PL		4431740	2/14/84		435	252.33	
PL		4440859	4/3/84		435	172.3	

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
PL		0006694	1-9-80	EPA	C12P	21/02		
PL		0037255	10-7-81	EPA	C07K 103/52			
PL		0037723	10-14-81	EPA	C12N 15/00			
PL		0040466	11-25-81	EPA	C12N 15/00			
PL		0070632	1-26-83	EPA	C12N 15/00			

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

EXAMINER	DATE CONSIDERED
Patricia Cano	9/21/89

*EXAMINER Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication

Form PTO-FB-D230 (8-83)
(also form PTO-1449)

Patent and Trademark Office · U.S. DEPARTMENT of COMMERCE

OMB No. 0651-0011 (12/8

INFORMATION DISCLOSURE CITATION FOR PATENT *(Use several sheets if necessary)*	ATTY. DOCK ID. P-6315- 934	Serial Number S. 739,123
	Patent Owner	
	~~Issue~~ Date F. 5/29/85	GROUP 185

U.S. PATENT DOCUMENTS PAGE 2 OF 2

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
BV		0 0 9 0 4 3 3	10-5-83	EPA	C12N	15/00		
BV		0 0 6 0 0 5 7	9-15-82	EP	C12N	15/00		
BV		1 4 6 4 8 2 B	10-14-80	Denmark	C12P	21/02		

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

EXAMINER	DATE CONSIDERED
CARSON	9/21/89

*EXAMINER Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication

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FIDELMAN AND WOLFFE
P.O.BOX 18218
WASHINGTON, DC 20036-8218

NOTICE OF ALLOWANCE AND ISSUE FEE DUE

			EXAMINER AND GROUP ART UNIT		DATE MAILED
06/739,123	05/29/85	032	CARSON, P	185	09/25/89

MARKUSSEN, JAN

DNA-SEQUENCE ENCODING BIOSYNTHETIC INSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

	TYPE	SERIES CODE	ART UNIT	SMALL ENTITY	FEE DUE	DATE DUE
1	104MF2196	530-303.000	W34	UTILITY NO	$620.00	12/26/89

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED.

THE ISSUE FEE MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED.

HOW TO RESPOND TO THIS NOTICE:

I. Review the SMALL ENTITY Status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the Status is changed, pay twice the amount of the FEE DUE shown above and notify the Patent and Trademark Office of the change in status, or

B. If the Status is the same, pay the FEE DUE shown above.

If the SMALL ENTITY is shown as NO:

A. Pay FEE DUE shown above, or

B. File verified statement of Small Entity Status before, or with, payment of 1/2 the FEE DUE shown above.

II. Part B of this notice should be completed and returned to the Patent and Trademark Office (PTO) with your ISSUE FEE. Even if the ISSUE FEE has already been paid by a charge to deposit account, Part B should be completed and returned. If you are charging the ISSUE FEE to your deposit account, Part C of this notice should also be completed and returned.

III. All communications regarding this application must give series code (or filing date), serial number and batch number. Please direct all communications prior to issuance to Box ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees.

PATENT AND TRADEMARK OFFICE COPY

PTOL-85 (REV 12-88)(OMB Clearance is pending)

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PART B - ISSUE FEE TRANSMITTAL

MAILING INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE. Blocks 2 through 6 should be completed where appropriate. All further correspondence including the Issue Fee Receipt, the Patent, advanced orders and notification of maintenance fees will be mailed to addressee entered in Block 1 unless you direct otherwise, by: (a) specifying a new correspondence address in Block 3 below; or (b) providing the PTO with a separate "FEE ADDRESS" for maintenance fee notifications with the payment of Issue Fee or thereafter. **See reverse for Certificate of Mailing.**

1. CORRESPONDENCE ADDRESS

MAIL ROOM DEC 18 1989 PAT. & TRADEMARK OFF.

2. INVENTOR(S) ADDRESS CHANGE (Complete only if there is a chang

INVENTOR'S NAME

Street Address

City, State and ZIP Code

CO-INVENTOR'S NAME

Street Address

City, State and ZIP Code

☐ Check if additional changes are on reverse side

SERIES CODE/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED

First Named Applicant:

TITLE OF INVENTION: A SEQUENCE ENCODING BOVINE PROINSULIN PRECURSORS AND PROCESS FOR PREPARING THE INSULIN PRECURSORS AND HUMAN INSULIN

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
	530-350.000	W34	UTILITY	NO	$620.00	

060 01/16/90 06739123 1 142 620.00 CK

3. Further correspondence to be mailed to the following:

Morris Fidelman
FIDELMAN & WOLFFE
P.O. Box 18218
Washington, D.C. 20036

4. For printing on the patent front page, list the names of not more than 3 registered patent attorneys or agents OR alternatively, the name of a firm having as a member a registered attorney or agent. If no name is listed, no name will be printed.

1 Morris Fidelman
2 Franklin D. Wol
3

DO NOT USE THIS SPACE

5. ASSIGNMENT DATA TO BE PRINTED ON THE PATENT (print or type)

(1) NAME OF ASSIGNEE: Novo Industri A/S

(2) ADDRESS: (City & State or Country) Novo Alle, DK-2880, Bagsvaerd

(3) STATE OF INCORPORATION, IF ASSIGNEE IS A CORPORATION: DENMARK

A. ☐ This application is NOT assigned.

☒ Assignment previously submitted to the Patent and Trademark Office.

☐ Assignment is being submitted under separate cover. Assignments should be directed to Box ASSIGNMENTS.

PLEASE NOTE: Unless an assignee is identified in Block 5, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the PTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

6a. The following fees are enclosed:

☒ Issue Fee ☒ Advanced Order - # of Copies 10 (Minimum c

6b. The following fees should be charged to:

DEPOSIT ACCOUNT NUMBER (Enclose Part C)

☐ Issue Fee ☐ Advanced Order - # of Copies (Minimum c

☐ Any Deficiencies in Enclosed Fees

The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Issue Fee to the application identified above.

(Signature of party in interest of record)	(Date)
Morris Fidelman	12-21-

NOTE: The Issue Fee will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the Patent and Trademark Office.

TRANSMIT THIS FORM WITH FEE-CERTIFICATE OF MAILING ON REVERSE

(if pending)

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FIG. 1

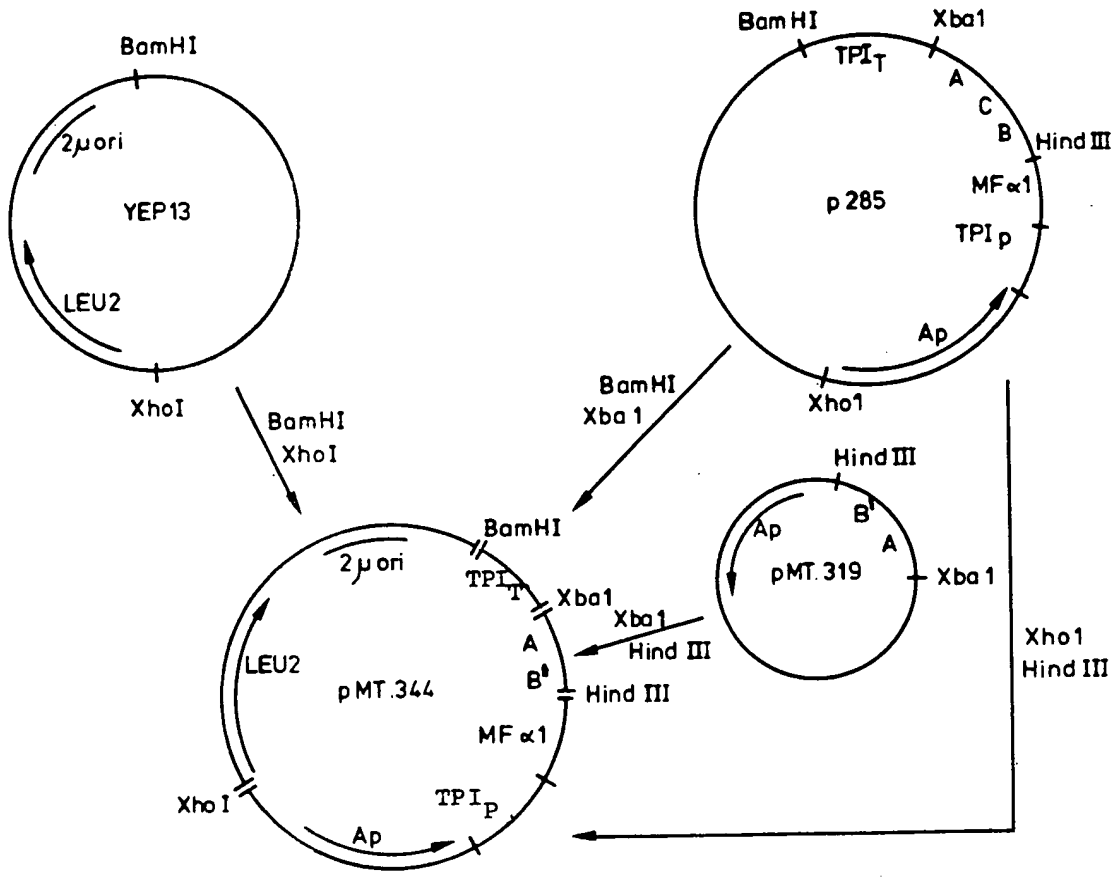
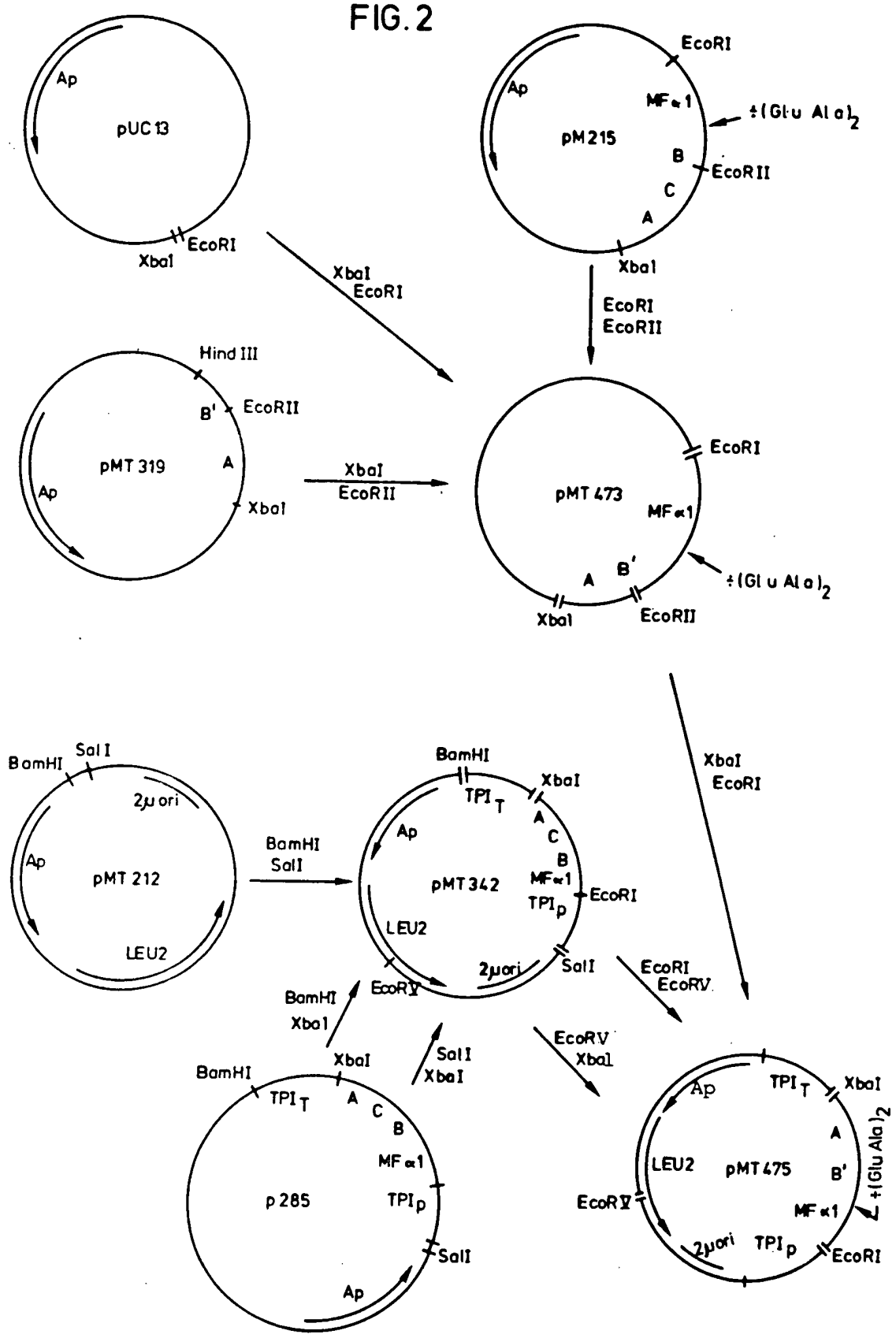


FIG. 2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XbaI
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SalI
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BamHI
XbaI
SalI
XbaI
EcoRV
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BamHI
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FIG.3

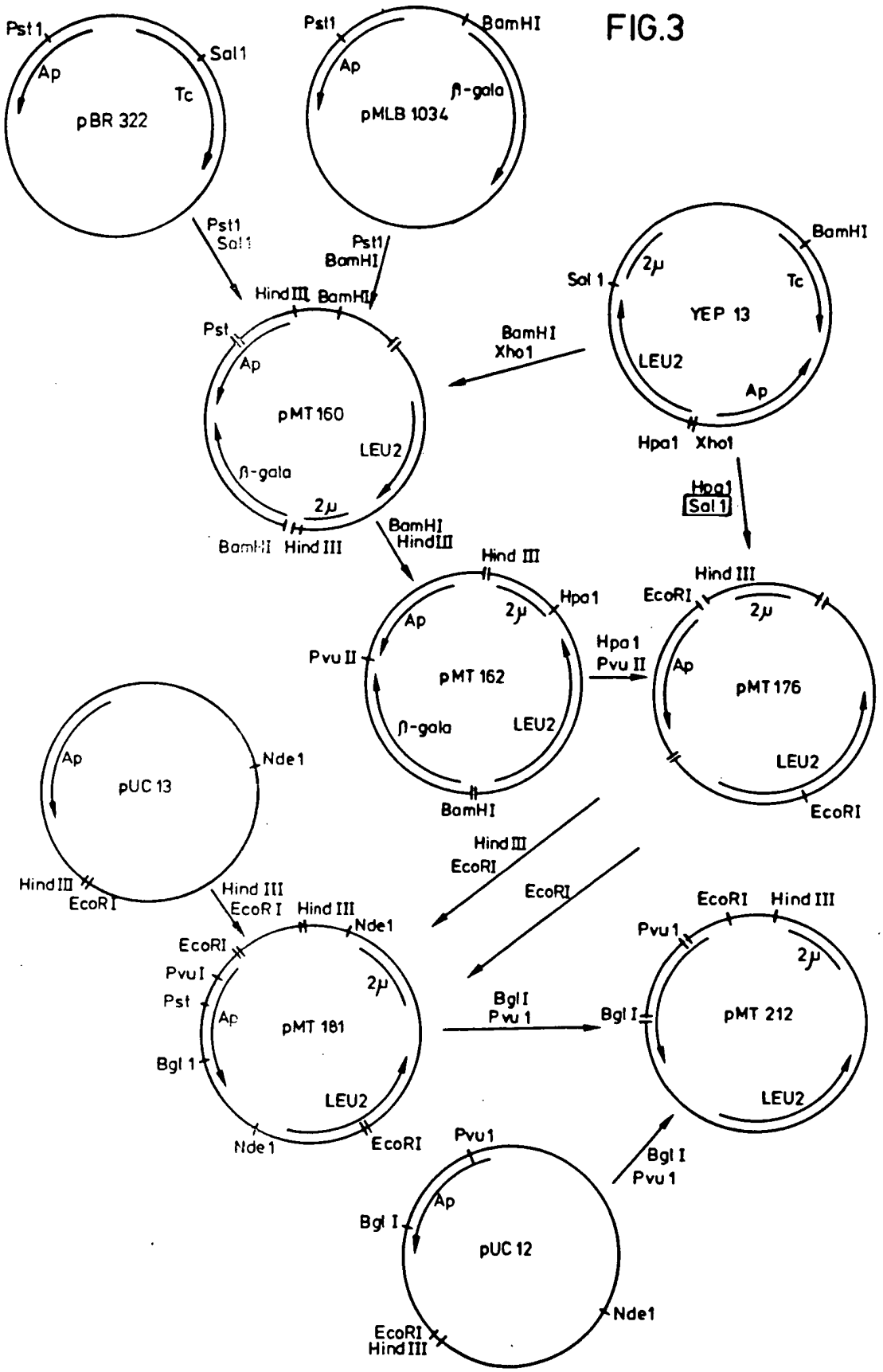


FIG. 4

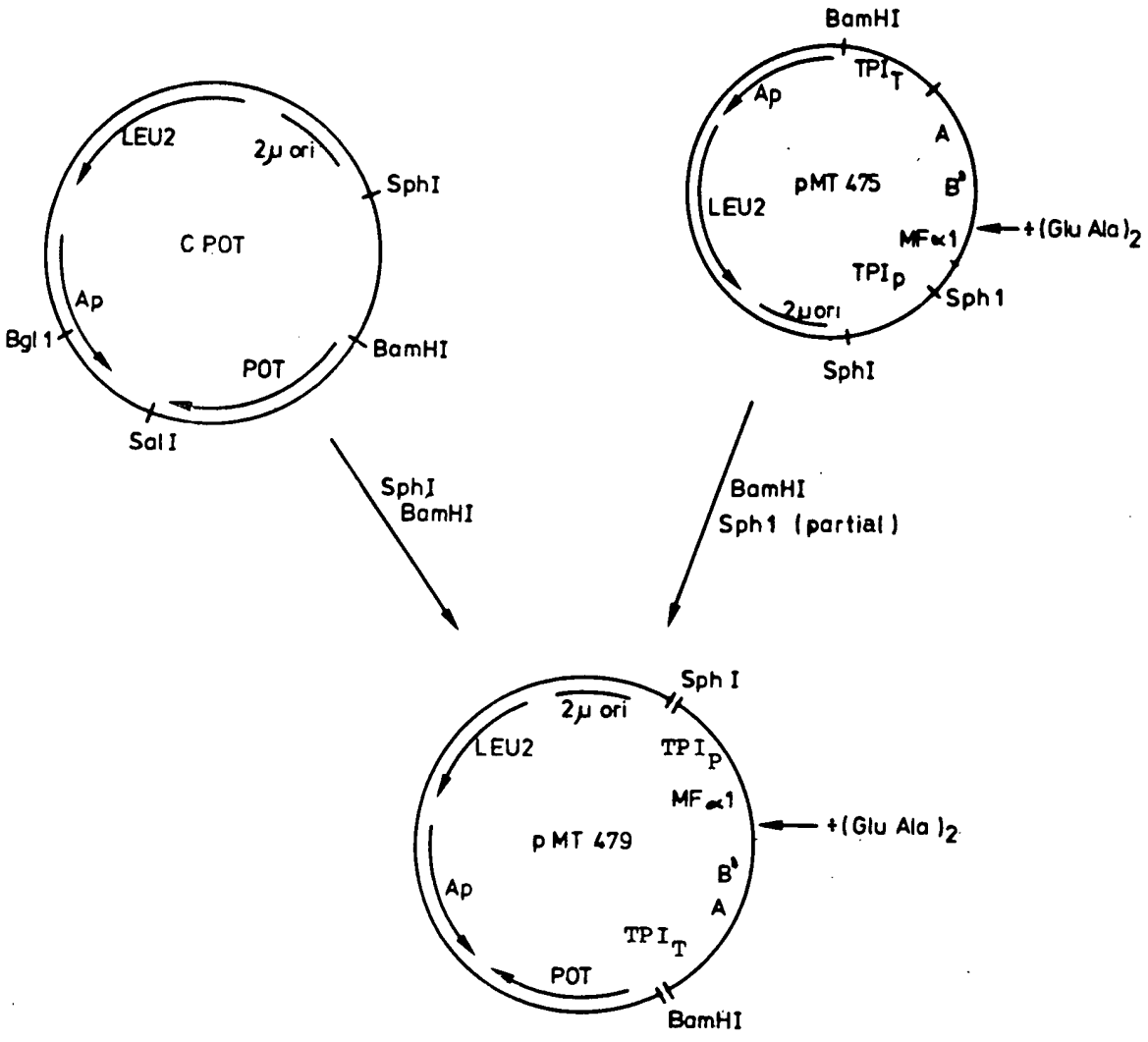


FIG. 5

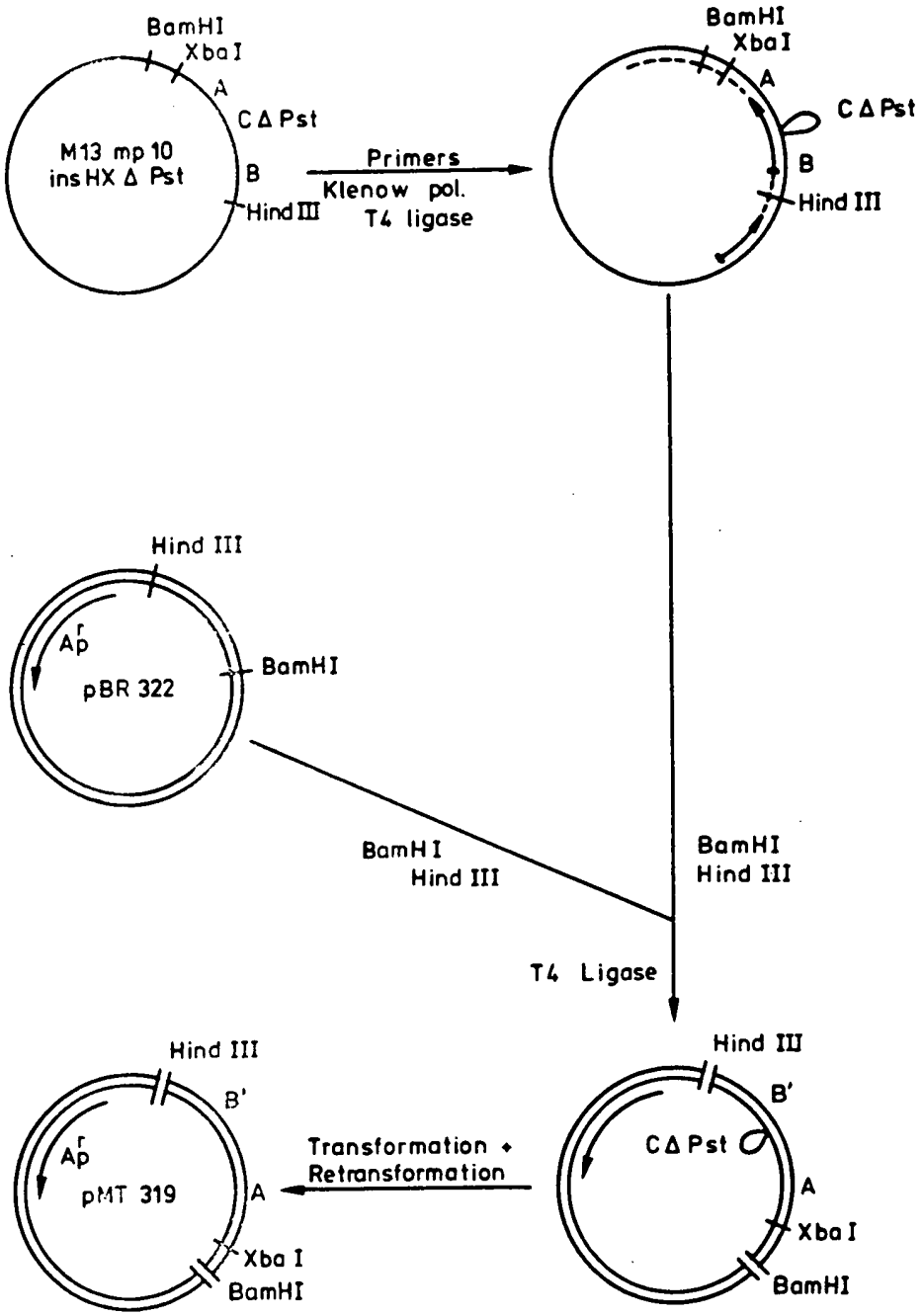


FIG.6

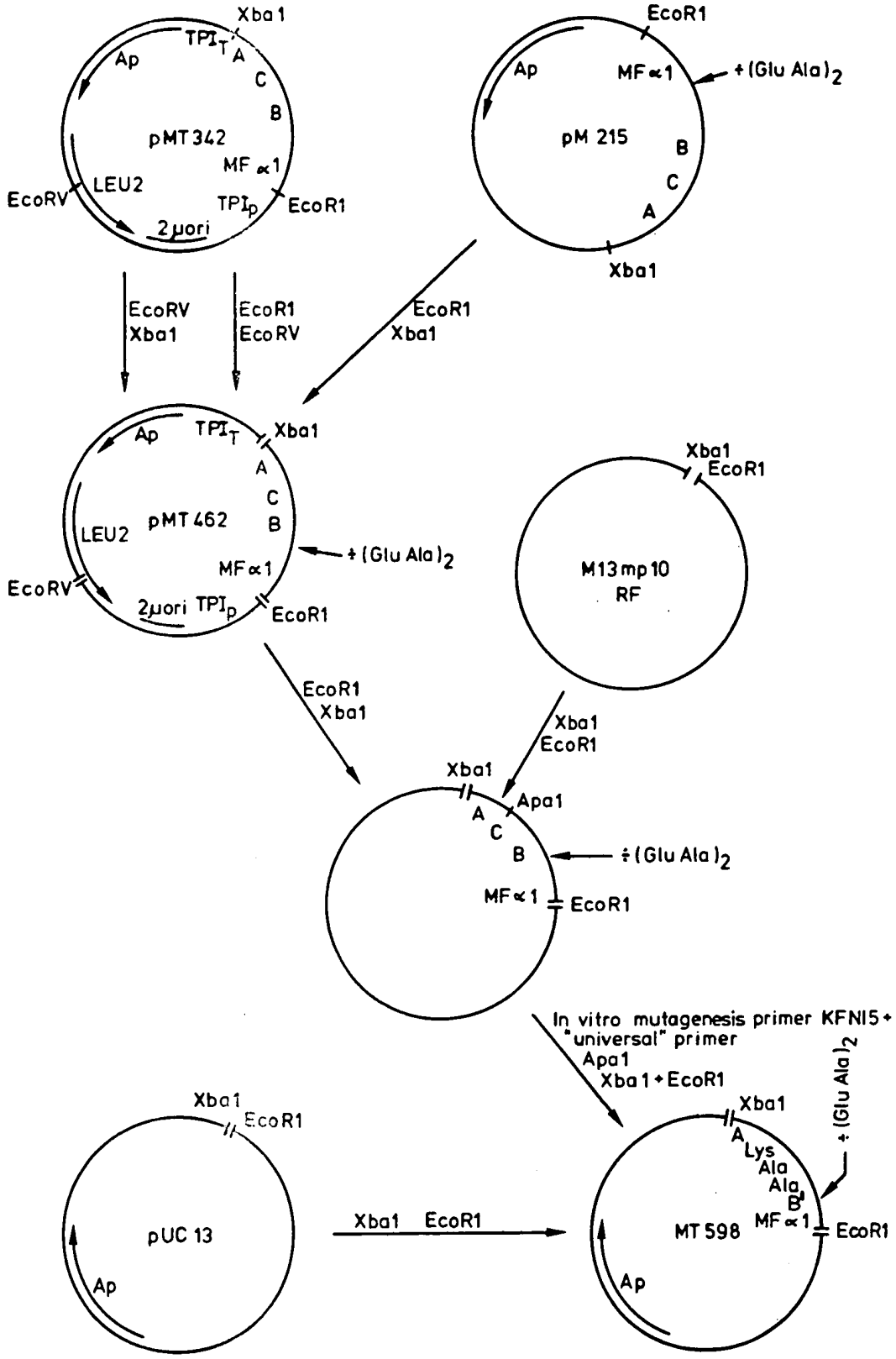


FIG. 7

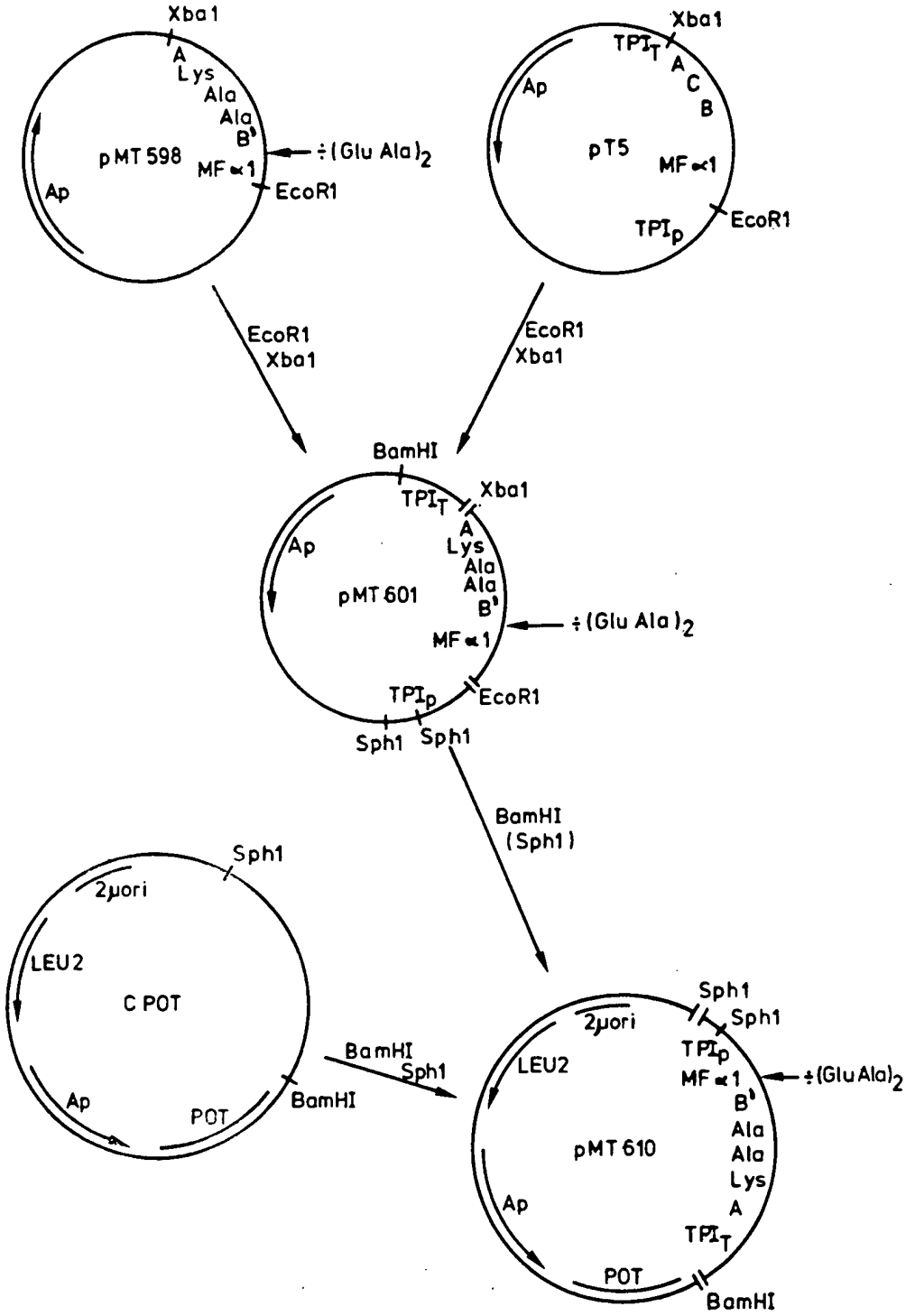


FIG. 8

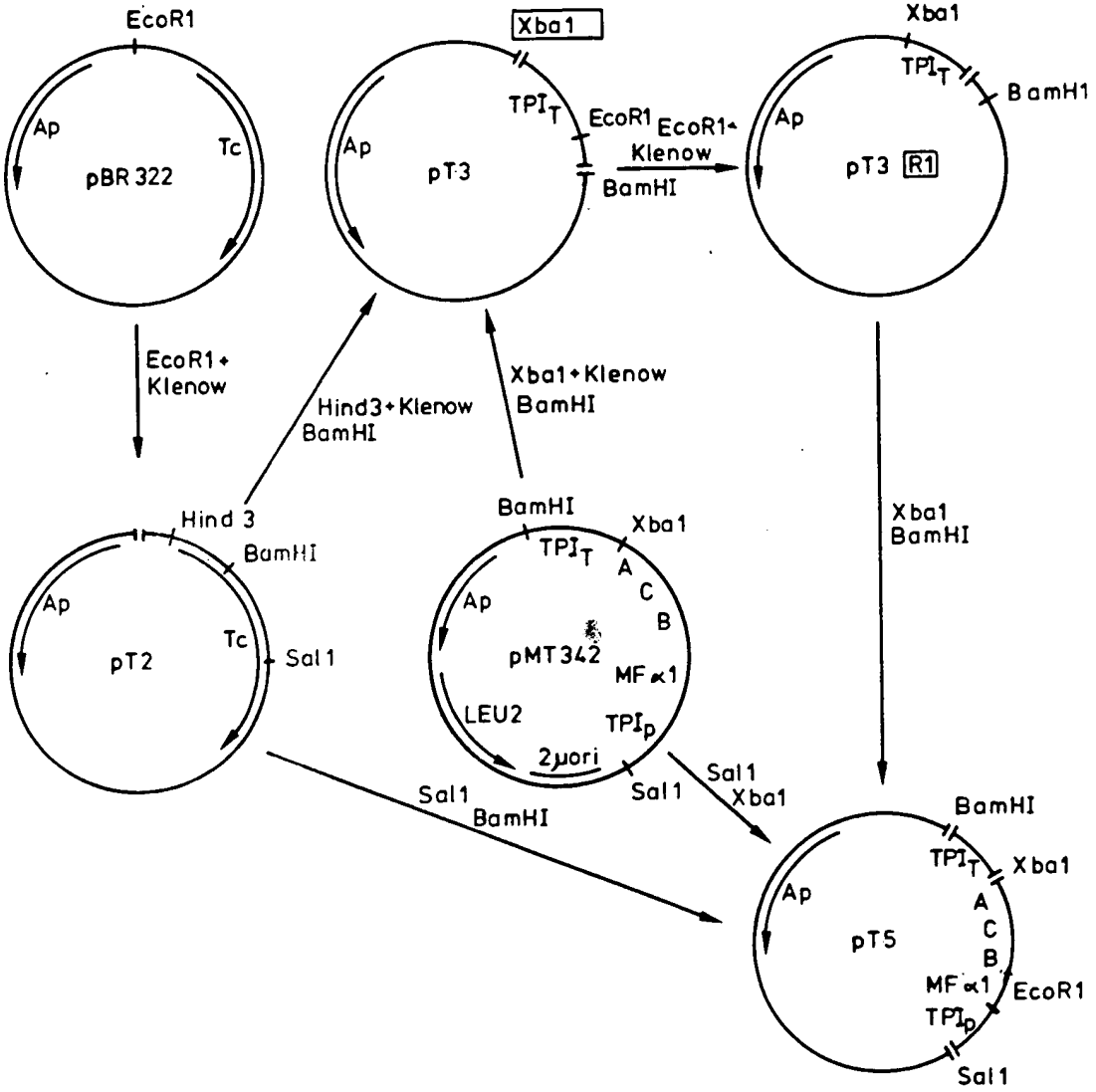
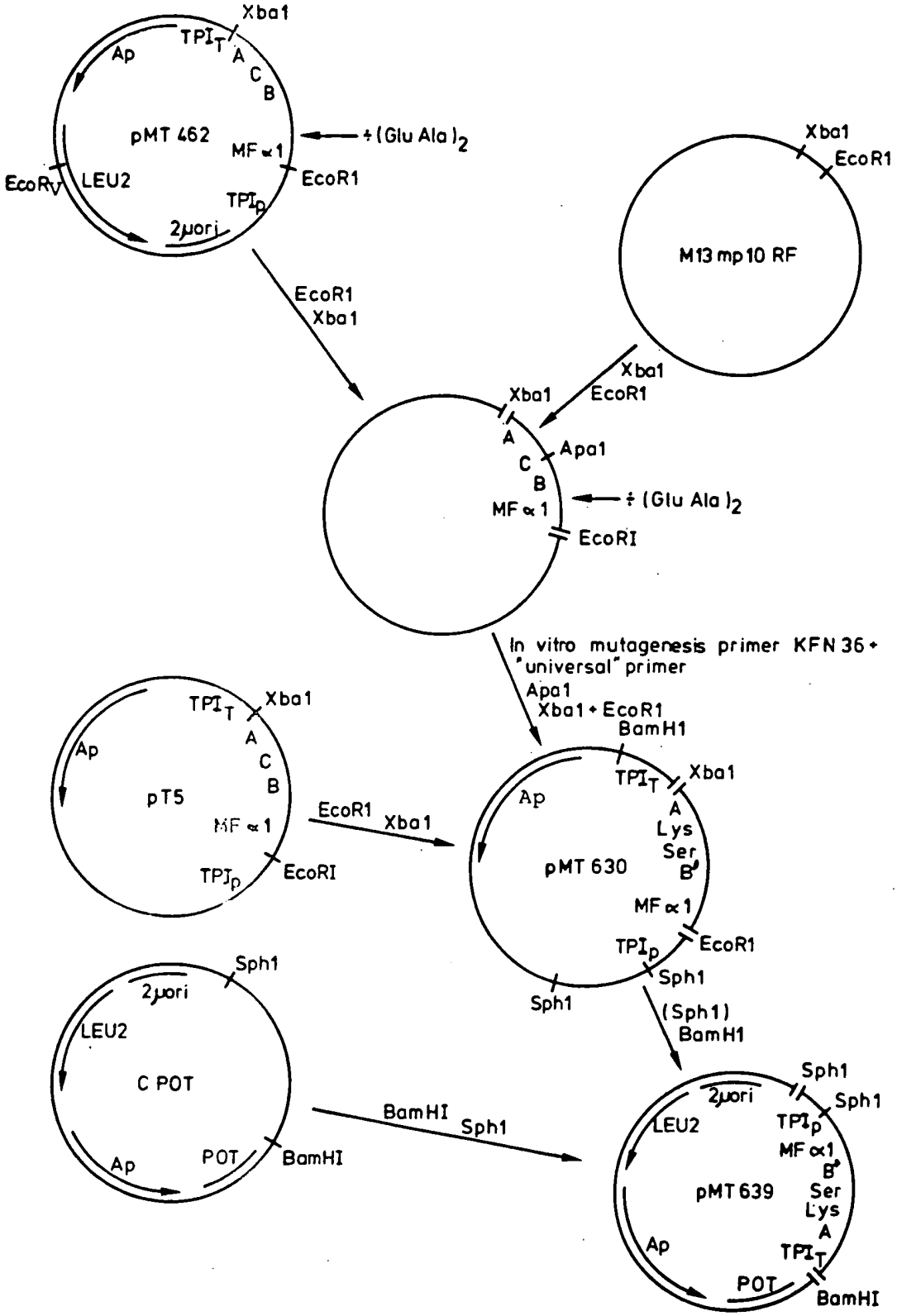


FIG. 9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Xba1
A
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C
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EcoRI
In vitro mutagenesis primer KFN 36 + "universal" primer
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Xba1 + EcoR1
BamH1
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Xba1
Ap
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C
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EcoRI
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PTO UTILITY GRANT

Paper Number 13

The United States of America

The Commissioner of Patents and Trademarks

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

Therefore, this

United States Patent

Grants to the person or persons having title to this patent the right to exclude others from making, using or selling the invention throughout the United States of America for the term of seventeen years from the date of this patent, subject to the payment of maintenance fees as provided by law.

PATENT AND TRADEMARK OFFICE * UNITED STATES OF AMERICA *

Acting Commissioner of Patents and Trademarks

Attest

AT ALLOWA

FIGURES DRWGS.	CLAIMS	CLASS
8	32	53

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